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UNITED STATES DISTRICT COURT
NORTHERN DISTRICT OF CALIFORNIA

FLUIDIGM CORPORATION, a Delaware corporation; and FLUIDIGM CANADA INC., a foreign corporation, Plaintiffs, v. IONPATH, INC., A Delaware corporation, Defendant.	Case No. 3:19-cv-05639 **FIRST AMENDED COMPLAINT FOR PATENT INFRINGEMENT AND INTENTIONAL INTERFERENCE WITH CONTRACTUAL RELATIONS** **JURY TRIAL DEMANDED** **Judge: Honorable William Alsup** **Complaint filed: September 6, 2019**

Plaintiffs, Fluidigm Corporation and Fluidigm Canada Inc. (collectively, "Fluidigm"), make the following allegations in support of their First Amended Complaint against Defendant, IONpath, Inc. ("IONpath"):

THE NATURE OF THE ACTION

1. This is an action for intentional interference with contractual relations and patent infringement arising out of IONpath's deliberate, willful, and ongoing infringement of Fluidigm's well-established rights.

2. Fluidigm provides innovative tools for biotechnology research including, for example, mass cytometry systems and methodologies that are used by customers such as leading academic, governmental, and private institutions worldwide.

3. Fluidigm is and has become an industry leader, in part, through its careful and extensive research, development, investment, and protection of its intellectual property rights including, among others, certain patent families dating back to at least 2004, as well as through the employ of, and consulting with, leading scientists in fields related to mass cytometry.

4. Consultants who were under contract with Fluidigm, and while retained by Fluidigm, formed a directly competing entity – IONpath – in partnership with other individuals. Even more distressing, the new IONpath enterprise has been developing a directly competing product to Fluidigm's mass cytometry systems requiring the use and implementation of Fluidigm's patented methodologies.

5. Indeed, Fluidigm learned that IONpath was using Fluidigm's proprietary antibodies and reagents to test a mass cytometry system that IONpath was purportedly developing. Concerned that IONpath was infringing upon Fluidigm's rights, and that IONpath was encouraging Fluidigm's customers to breach their contracts with Fluidigm regarding the antibodies and reagents, Fluidigm repeatedly contacted IONpath in an effort to persuade IONpath to voluntarily cease its improper and unlawful conduct.

6. Instead of heeding Fluidigm's warnings, and ceasing its conduct, in September 2018, IONpath published an article and issued press releases touting the results IONpath obtained using Fluidigm's patented methodologies. Recently, IONpath began aggressively marketing the launch date of its new and infringing product set for late 2019.

7. IONpath's publications establish that not only had IONpath been developing its mass cytometry system employing Fluidigm's antibodies and reagents, but also wholesale adopted and is using Fluidigm's patented methods and systems – and is selling its infringing products and systems in the marketplace.

8. IONpath's conduct, including its refusal to cease its infringing conduct, leaves Fluidigm with no option but to proceed with this lawsuit in order to prevent IONpath from continuing its improper, wrongful, widespread, and willful infringement of Fluidigm's patented technologies. In addition to seeking the cessation of IONpath's wrongful conduct, it also seeks redress for IONpath's past infringement and tortious interference with Fluidigm's contractual relationships with its customers.

PARTIES

9. Fluidigm Corporation is a Delaware corporation with a principal place of business located at 7000 Shoreline Court, Suite 100, South San Francisco, California 94080.

10. Fluidigm Canada Inc. is an Ontario corporation organized under the laws of Ontario with its principal place of business at 1380 Rodick Rd., Markham, Suite 100, Ontario, L3R 4G5 Canada. Fluidigm Canada Inc. is a wholly owned subsidiary of Fluidigm Sciences Inc., a Delaware corporation, which is a wholly owned subsidiary of Fluidigm Corporation.

11. On information and belief, IONpath is a Delaware corporation with a principal place of business within this judicial district at 960 O'Brien Dr., Menlo Park, California 94025.

JURISDICTION AND VENUE

12. This Court has subject matter jurisdiction pursuant to 28 U.S.C. §§ 1331 and 1338(a) because this is an action for, in part, patent infringement arising under the patent laws of the United States, 35 U.S.C. § 1, *et seq*., including §§ 271 and 281. This Court has subject matter jurisdiction over the tortious interference claim pursuant to 28 U.S.C. § 1367(a).

13. On information and belief, IONpath is subject to this Court's specific and general personal jurisdiction, pursuant to due process and/or the California Long Arm Statute.

14. Venue is proper in this District under 28 U.S.C. §§ 1391(b) and 1391(c) because IONpath resides and does substantial business in this District, and has committed acts of patent infringement and tortious interference in this District. Venue is also proper in this District under 28 U.S.C. § 1400(b) because IONpath is subject to personal jurisdiction in this District, has a principal place of business in this District, has committed and continues to commit acts of infringement in this District, and a substantial part of the events giving rise to the claims occurred in this District.

BACKGROUND

I. FLUIDIGM'S INDUSTRY LEADING ROLE IN MASS CYTOMETRY

15. Fluidigm is a leading provider of cutting-edge tools, systems, and methods for biotechnology research including, among other things, microfluidics systems, suspension-based mass cytometry and Imaging Mass Cytometry™. Fluidigm's customers include, for example,

leading academic, government, pharmaceutical, biotechnology, and plant and animal research laboratories worldwide.

16. Fluidigm's proprietary technologies enable the scientific discovery and investigation in critical areas of research, provide meaningful insights into health and disease, and accelerate the development of effective therapies including, for example, in the areas of cancer, immunology and immunotherapy.

17. Mass cytometry is a technique for investigating structures and biomarkers on the cellular level, including intracellular features. Fluidigm's proprietary CyTOF®, Helios™ and Hyperion™ mass cytometry technologies allow for the interrogation of more than 40 markers simultaneously on thousands of individual cells, which can enable the identification of new cell types, functions, and biomarkers indicative of different disease states and/or responsiveness to therapeutic treatments.

18. Fluidigm's proprietary Maxpar® reagents provide critical state-of-the-art labelling tools for identification of targets using Fluidigm's proprietary mass cytometry techniques, methods, and systems.

19. Foundational aspects of mass cytometry technology were developed, in part, by Dr. Scott Tanner as an improvement over prior fluorescence-based techniques. Specifically, while fluorescence-based flow cytometry techniques only identified a limited number of markers because of spectral overlap of fluorophores, mass cytometry uses specialized reagents – metal tags attached to specific antibodies – that can be quantified using high resolution mass spectrometry. The ability to use stable metal isotopes not normally found in biological systems, with target-specific antibodies, in a transient single cell, allows for the quantification of multiple

parameters in a cell, both on a cell-by-cell basis, as well as across potentially thousands of cells. The invention of this technique involved leveraging of innovations in areas including, for example, mass spectrometry and biotechnology, specifically: providing ionized and atomized elemental tags from individual cells, time-of-flight technology and ion optics for detecting a plurality of element tags from individual cells, and polymer and metal ligand chemistry.

20. Dr. Tanner and others, including Drs. Vladimir I. Baranov and Dmitry R. Bandura, further developed the proprietary technique and, based upon their inventions, certain patent applications were filed. Shortly thereafter, the inventors started their company DVS Sciences, Inc. ("DVS"), and licensed certain aspects of their patent applications. Fluidigm acquired DVS in 2014, purchased the proprietary technology – including the patent rights relating to the technique, systems, and methods, and continued to work on and develop the technology, systems and methods for mass cytometry, including the CyTOF® mass cytometer, the next generation Helios™ mass cytometer released in 2015, and the Hyperion™ Imaging System released in 2017.

21. While at DVS, Dr. Vladimir I. Baranov also collaborated with the University of Toronto to further develop CyTOF® technologies, including specialized reagents for mass cytometry techniques. As part of this work, patent applications for certain reagents and technologies were filed which were assigned to DVS and subsequently assigned to Fluidigm. Fluidigm has continued to work on and develop the technology, systems and methods for mass cytometry, including the Maxpar® antibodies and reagents for use with these systems.

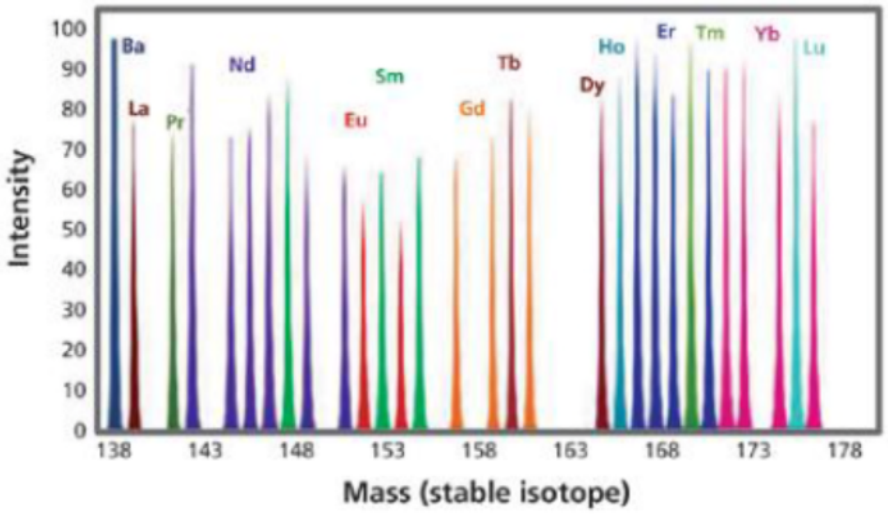
22. In performing a mass cytometry analysis using Fluidigm's patented methodologies, the sample of interest (e.g., a suspension of cells or tissue sample) is labelled by

introducing reagents comprising metal tags attached to antibodies that are specific to targets of interest within the sample, a process often referred to as "staining" of the sample. The antibodies specifically bind to the targets of interest if present in the sample, which may be specific cellular components, such that the targets are labelled with the particular metal tag connected to the bound antibody. The metal tags may be different elemental metal isotopes (such as lanthanide isotopes) that differ from each other in their varying weights.

23. The reagents used for labelling provide a plurality of antibodies with metal tags, with each labelling antibody that is specific for a particular target being attached to a metal tag having a different weight than the metal tags of other labelling antibodies that are specific for other targets. A washing step may be performed to remove any unreacted reagent that did not bind to a target, such that generally only those labels that find and bind to their targets remain in the sample.

24. To quantitatively analyze the labelled sample, a procedure is performed to release the metal tags from the sample, and the ionized metal tags are provided to a device known as a mass spectrometer, which is capable of detecting and measuring the mass to charge ratio of the resulting ions. Because the metal tags have different weights (i.e., a different weight for each target-specific antibody), the mass spectrometer is capable of detecting and distinguishing the different metal tags used for labelling, meaning that information related to the presence or absence of the antibody-bound targets in the sample is obtained. The output from this detection can be analyzed and processed to provide multiplex information at the subcellular level, including comprehensive functional and phenotypic characterization of complex systems at the single-cell level of samples containing potentially thousands of cells.

25. Using Fluidigm's proprietary technique and technology, an example output of isotope signals generated from single cells is illustrated below for various metal isotopes:



26. In addition, Fluidigm is the leading industry provider of labelling antibodies and other reagents for use in mass cytometry. Specifically, Fluidigm offers the Maxpar® antibodies and reagents, which have been developed specifically for use with Fluidigm's proprietary mass cytometry technology. The Maxpar® antibodies and reagents provide for the labelling of samples with antibodies conjugated to metal tags to allow for multiplex imaging (i.e. the evaluation of multiple targets in the sample), and thus are an important component in state of the art mass cytometry analysis.

27. Fluidigm's mass cytometry products have been critical for breakthrough discoveries in numerous research areas including, for example, in biomarker screening,

immunotherapy, immunology, immunophenotyping, infectious disease/microbiology, inherited disease detection, neurology, and oncology.

II. FLUIDIGM'S ASSERTED PATENTS

28. Fluidigm has carefully protected its inventions and cutting-edge technologies with a range of intellectual property rights. In particular, the U.S. Patent and Trademark Office ("the Patent Office") has awarded Fluidigm numerous patents covering its extremely valuable technologies.

A. '386 Patent

29. Fluidigm is the assignee of all right, title, and interest in and to U.S. Patent No. 10,180,386 (the "'386 Patent"), titled "Mass Spectrometry Based Multi-Parametric Particle Analyzer." The Patent Office duly and properly issued the '386 Patent on January 15, 2019. A true and correct copy of the '386 Patent is attached hereto as Exhibit A.

30. Fluidigm is the owner of all right, title, and interest in the '386 Patent, including all rights to pursue and collect damages for past, present, and future infringement of the '386 Patent.

31. The Patent Office carefully examined the '386 Patent, and its associated patent family, over a period of years going back to 2004. In doing so, the Patent Office found that the inventions described and claimed in the '386 Patent are both new and not obvious in light of prior patents, publications, and other art, and determined that the claimed systems and methods are inventive and patentable.

32. The claims of the '386 Patent are generally directed to methods for analyzing cells by mass spectrometry. For example, claim 1 of the '386 Patent recites:

> A method of sequentially analyzing single cells by mass spectrometry, comprising:
>
> providing a sample containing a plurality of tagged cells tagged with a plurality of tagged antibodies, wherein each of the tagged antibodies is specific for a different analyte, and wherein each of the tagged antibodies is tagged with an elemental tag comprising a lanthanide or noble metal;
>
> vaporizing, atomizing, and ionizing multiple elemental tags from a single first cell of the plurality of tagged cells;
>
> detecting, using mass spectroscopy, the elemental composition of the first cell by detecting a transient signal of the multiple vaporized, atomized, and ionized elemental tags of the first cell;
>
> vaporizing, atomizing, and ionizing multiple elemental tags from a single second cell of the plurality of tagged cells; and
>
> detecting, using mass spectrometry, the elemental composition of the second cell by detecting a transient signal of the multiple vaporized, atomized, and ionized elemental tags of the second cell, wherein the transient signal associated with the first cell and the transient signal associated with the second cell are detected sequentially.

33. The ‘386 Patent describes that the technology disclosed therein overcomes deficiencies in prior cell analysis methods, such as the issues with spectral overlap encountered in flow cytometry, to provide improved analysis of intracellular and cell surface characteristics. ‘386 Patent at column 2, lines 11-45, and Examples 1-13.

34. Importantly, the technology and methods claimed in and covered by the ‘386 Patent are unique and novel and permit individuals to conduct extremely valuable analysis on a cellular level. The ‘386 Patent includes important insights including, among other things, the use of mass spectrometry on biological materials, an understanding of how to use the biological materials (antibodies targeting analytes), time of flight analysis to allow for the separation of the

lanthanide or noble metal tag by weight, and the understanding of how to use the different metal isotopes coupled with antibodies as reagents.

35. The '386 Patent, and its patent family, relate back to a provisional patent application filed on March 25, 2004, as well as, among others, U.S. Patent Nos. 9,952,134 (the "'134 Patent") and 7,479,630 (the "'630 Patent").

36. On information and belief, IONpath had notice of the '386 Patent and its patent family. Specifically, two of IONpath's founders applied for a patent in 2011, publication number US2012/0077714 A1, citing to, among other sources, the '630 Patent, to which the '386 claims priority and which contains the same disclosure as the '386 patent. The two IONpath founders again referenced Fluidigm's related '630 Patent in their 2013 list of references submitted to the Patent Office.

B. '104 Patent.

37. Fluidigm Canada is the assignee of U.S. Patent No. 10,072,104 (the "'104 Patent"), titled "Polymer Backbone Element Tags." The Patent Office duly and properly issued the '104 Patent on September 11, 2018. A true and correct copy of the '104 Patent is attached hereto as Exhibit B.

38. Fluidigm Canada is the owner of all right, title, and interest in and to the '104 Patent, including all rights to pursue and collect damages for past, present, and future infringement of the '104 Patent.

39. The Patent Office found that the inventions described and claimed in the '104 Patent are both new and not obvious in light of prior patents, publications, and other art, and determined that the claimed systems and methods are inventive and patentable.

40. The claims of the ‘104 Patent are generally directed to methods for analysis of a sample. For example, claim 1 of the ‘104 Patent recites:

> A method for the analysis of an analyte in a sample, comprising:
>
> (i) incubating an element tagged affinity reagent with an analyte, the element tagged affinity reagent comprising an affinity reagent tagged with an element tag, the element tag comprising a linear or branched polymer having multiple metal-binding pendant groups, wherein each pendant group includes at least one metal atom or is capable of binding at least one metal atom, and wherein the affinity reagent specifically binds with the analyte, wherein the analyte is located within or on an intact cell;
>
> (ii) separating unbound element tagged affinity reagent from bound element tagged affinity reagent; and
>
> (iii) analyzing the element tag bound to the affinity reagent attached to the analyte of the intact cell by atomic spectroscopy, wherein analyzing occurs without prior acidification of the sample.

41. The ‘104 Patent describes that the mass spectrometry technology disclosed and claimed therein provides extremely valuable improvements over prior methods including UV vis spectroscopy, HPLC, flow cytometry, ligand binding assays, and others, by allowing for multiplexing (determining the presence of multiple targets in a sample), improved sensitivity and improved selectivity. ’102 Patent at column 1, lines 35-60.

42. Fluidigm provided IONpath notice of the ‘104 Patent on September 24, 2018, as discussed in more detail below.

C. ‘698 Patent

43. Fluidigm is the assignee of all right, title, and interest in and to U.S. Patent No. 10,436,698 (the “‘698 Patent”), titled “Mass Spectrometry Based Multi-Parametric Particle

Analyzer." The Patent Office duly and properly issued the '698 Patent on October 8, 2019. A true and correct copy of the '698 Patent is attached hereto as Exhibit C.

44. Fluidigm is the owner of all right, title, and interest in the '698 Patent, including all rights to pursue and collect damages for past, present, and future infringement of the '698 Patent.

45. The Patent Office carefully examined the '698 Patent, and its associated patent family (including the '386 Patent discussed above), over a period of years going back to 2004. In doing so, the Patent Office found that the inventions described and claimed in the '698 Patent are both new and not obvious in light of prior patents, publications, and other art, and determined that the claimed systems and methods are inventive and patentable.

46. The claims of the '698 Patent are generally directed to systems for analyzing cells in a sample by mass spectrometry. For example, claim 1 of the '698 Patent recites:

> A system for sequentially analyzing single cells in a sample by mass spectrometry,
>
> wherein the sample comprises a plurality of tagged cells tagged with a plurality of tagged antibodies, wherein each of the plurality of tagged antibodies is specific for a different analyte, and wherein each of the plurality of tagged antibodies is tagged with an elemental tag comprising a lanthanide or noble metal;
>
> wherein the system comprises:
>
> a first device to vaporize, atomize, and ionize multiple elemental tags from a single first cell of the plurality of tagged cells and multiple elemental tags from a single second cell of the plurality of tagged cells; and
>
> a second device to detect, by mass spectrometry, lanthanides and/or noble metals of the single first cell by detecting a transient signal of the multiple vaporized, atomized, and ionized elemental tags of the single first cell, and lanthanides and/or noble metals of the single second cell by detecting a transient signal of the multiple vaporized, atomized, and ionized elemental tags of the

> single second cell, wherein the transient signal associated with the single first cell and the transient signal associated with the single second cell are detected sequentially.

47. The ‘698 Patent describes that the technology disclosed therein overcomes deficiencies in prior cell analysis systems, such as the issues with spectral overlap encountered in flow cytometry systems, to provide improved analysis of intracellular and cell surface characteristics. ‘698 Patent at column 2, lines 14-47, and Examples 1-13.

48. Importantly, the technology and systems claimed in and covered by the ‘698 Patent are unique and novel and permit individuals to conduct extremely valuable analysis on a cellular level. The ‘698 Patent includes, among other things, important insights into systems and devices for performing mass spectrometry on biological materials, such as biological samples stained with tagged antibodies targeting analytes, and systems and devices to detect lanthanides and/or noble metals that use time of flight analysis to allow for the separation of the lanthanide and/or noble metal tags by weight.

49. The ‘698 Patent, and its patent family, relate back to a provisional patent application filed on March 25, 2004, as well as, among others, the ’134 and ’630 Patents.

50. On information and belief, IONpath had notice of the ‘698 Patent and its patent family. Specifically, two of IONpath’s founders applied for a patent in 2011, publication number US2012/0077714 A1, citing to, among other sources, the ‘630 Patent, to which the ‘698 claims priority and which contains the same disclosure as the ‘698 patent. The two IONpath founders again referenced Fluidigm’s related ‘630 Patent in their 2013 list of references submitted to the Patent Office.

III. FLUIDIGM CONSULTANTS FORM IONPATH

A. Dr. Garry Nolan

51. In March 2014, Dr. Nolan along with Drs. Angelo and Bendall and several other authors, published a technical report (the "2014 Technical Report") discussing their academic research on working with multiplexed ion beam imaging in the field of immunohistochemistry.

52. On May 16, 2014, Fluidigm entered into a Scientific Consulting Agreement ("Nolan Consulting Agreement") with Professor Garry Nolan ("Dr. Nolan"). Pursuant to the Nolan Consulting Agreement, Fluidigm retained Dr. Nolan for the purpose of assisting Fluidigm with "the development and applications of mass cytometry-based instrumentation, including associated reagents," defined in the Nolan Consulting Agreement as the "Field of Interest."

53. In the Nolan Consulting Agreement, Dr. Nolan agreed:

> [T]hat during the Term of this [Consulting] Agreement he will not directly or indirectly (i) provide any services in the Field of Interest to any other business or commercial entity, including without limitation research or other work for which any business or commercial entity has rights (e.g., patent rights, a right to a license, or a first or other right to negotiate a license), (ii) participate in the formation of any business or commercial entity in the Field of Interest, or (iii) solicit or hire away any employee or consultant of the Company.

Nolan Consulting Agreement at ¶ 5.

54. The Nolan Consulting Agreement contained appropriate carve-outs to allow and encourage Dr. Nolan to continue to work as an employee of the university of which he was employed as a professor. *Id.* The Nolan Consulting Agreement also limited its term to four years or upon earlier termination upon 30 day notice. *Id.* at ¶ 3.

55. The Nolan Consulting Agreement also required Dr. Nolan to disclose opportunities, as well as direct project and inventions, to Fluidigm in the Field of Interest. *Id.*

at ¶ 6. Importantly, the Nolan Consulting Agreement provided that Dr. Nolan "will use his best efforts (i) to disclose to the President of the Company, on a confidential basis, technology and product opportunities which come to the attention of [Dr. Nolan] in the Field of Interest, and any invention, improvement, discovery, process, formula, or method or other intellectual property relating to or useful in the Field of Interest (collectively "New Discoveries"), whether or not patentable or copyrightable, and whether or not discovered or developed by [Dr. Nolan], and (ii) with regard to any New Discoveries discovered or developed by [Dr. Nolan] at the Institution, to solicit or encourage the Institution to enter into an agreement with the Company to exclusively license such New Discovery to the Company." *Id.* at ¶ 6.

56. Where Dr. Nolan developed any "invention, improvement, discovery, process, formula, technique, method, trade secret, or other intellectual property" (collectively, "Inventions" or "Invention") while performing services under the Consultant Agreement, he agreed to "promptly and fully disclose" any Invention to Fluidigm. *Id.* at ¶ 7. And Dr. Nolan agreed to assign his rights to all such Inventions to Fluidigm. *Id.*

57. The Nolan Consulting Agreement also contained a confidentiality provision requiring Dr. Nolan to, in part, "not disclose, directly or indirectly, [Fluidigm's confidential information] to any third person or entity . . . ." *Id.* at ¶ 8.3.

58. Dr. Nolan worked under the Nolan Consulting Agreement with Fluidigm for over two and one-half years, until they mutually agreed to terminate the Nolan Consulting Agreement effective on December 31, 2016. At the time of the termination of the Nolan Consulting Agreement, Dr. Nolan understood, knew of, and Fluidigm reminded him of his confidentiality obligations owed to Fluidigm.

59. On September 16, 2014, shortly after Dr. Nolan entered into the Nolan Consulting Agreement, Dr. Nolan together with Drs. Angelo, Bendall, and Feinberg founded IONpath.

60. On information and belief, and while Dr. Nolan consulted with Fluidigm, IONpath and Dr. Nolan began working on directly competing technologies, as well as commercializing products, for pathology applications, including, eventually, IONpath's "MIBIscope" technology.

B. Dr. Sean Bendall

61. Dr. Sean Bendall co-founded IONpath in September 2014 and currently sits on its Board of Directors. Prior to that time, Fluidigm retained Dr. Bendall to serve as a Consultant. Specifically, Fluidigm and Dr. Bendall entered into a Consulting Agreement dated April 26, 2014 and again on April 26, 2015 ("Bendall Consulting Agreements").

62. Pursuant to the Bendall Consulting Agreements, Fluidigm retained Dr. Bendall "to provide the following services: consulting services relating to labeling of biological samples and conjugation of biomolecules (including but not limited antibodies and other affinity reagents) with metal isotopes for use in inductively-coupled plasma (ICP) based biological assays including, but not limited to, the analysis of single-cells, biological extracts, homogenates, and fluids." Bendall Consulting Agreements at Ex. A.

63. Dr. Bendall agreed that he "will not, during or subsequent to the term of this Agreement, use the Company's Confidential Information for any purpose whatsoever other than the performance of the Services on behalf of the Company or disclose the Company's Confidential Information to any third party." *Id.* at ¶ 2(b).

64. Dr. Bendall disclosed that he was "simultaneously and principally employed by and/or associated with Stanford University." *Id.* at ¶ 3(e). Dr. Bendall certified that "[e]xcept for Consultant's obligations to its fulltime employers," he "has no outstanding agreement or obligation that is in conflict with any of the provisions of this Agreement or that would preclude Consultant from complying with the provisions hereof, and further certifies that Consultant will not enter into any such conflicting agreement during the term of this Agreement." *Id.* at ¶ 4.

65. The Bendall Consulting Agreements provide that "Consultant is free to perform work as a consultant or employee for any other entity and/or person provided that such engagement does not create a conflict of interest with Consultant's obligations to Company." *Id.* at ¶ 7(b).

66. The Bendall Consulting Agreements were to expire on April 25, 2016 unless agreed otherwise in writing by both parties. *Id.* at ¶ 5(a). Dr. Bendall and Fluidigm extended the term of the Bendall Consulting Agreements twice to April 25, 2018.

67. On information and belief, and while Dr. Bendall consulted with Fluidigm, IONpath and Dr. Bendall began commercializing products for pathology applications, including, eventually, IONpath's "MIBIscope" technology.

IV. IONPATH'S MARKETING AND SALE OF ITS INFRINGING TECHNOLOGY

68. By at least February 2018, on information and belief, IONpath began contacting Fluidigm's customers for the express purpose of convincing Fluidigm's customers to use Fluidigm's proprietary Maxpar® antibodies and related reagents with IONpath's systems.

69. On or about February 7, 2018, Fluidigm put IONpath on notice that by inducing and seeking to convince customers to use the Maxpar antibodies and related reagents with IONpath's technology, IONpath was causing customers to breach the Sales and License Terms and Conditions ("Ts&Cs") each customer agreed to when using Fluidigm's proprietary Maxpar® antibodies and related reagents.

70. Specifically, Fluidigm identified, among others, that Fluidigm's customers had agreed to not: ". . . (iv) use any Fluidigm consumables or reagents with any non-Fluidigm instrument, device or system." Ts&Cs at § 3.2.

71. Fluidigm also expressed its concern that IONpath sought to employ former Fluidigm consultants and employees to foster an environment where those employees and consultants would be pressured to breach their confidentiality obligations owed to Fluidigm.

72. While IONpath denied any wrongdoing in two letters in February and March 2018, Fluidigm later learned that IONpath's founders Drs. Bendall and Angelo, even after the warning from Fluidigm, submitted a paper to the *Cell* journal on April 16, 2018, that admitted to using Fluidigm's Maxpar X8 Antibody labeling kit as a Critical Commercial Assay for IONpath's technology, in violation of the Ts&Cs.

73. Not only did the paper admit that IONpath knew that it and its founders breached the Ts&Cs but also encouraged Fluidigm's customers to similarly breach their Ts&Cs by using Fluidigm antibodies and related reagents with non-Fluidigm systems.

IONpath announces its infringing MIBIscope technology in 2018.

74. In the September 6, 2018 press release ("2018 Press Release"), IONpath celebrated the publication of Drs. Bendall and Angelo's work in the journal *Cell* detailing the

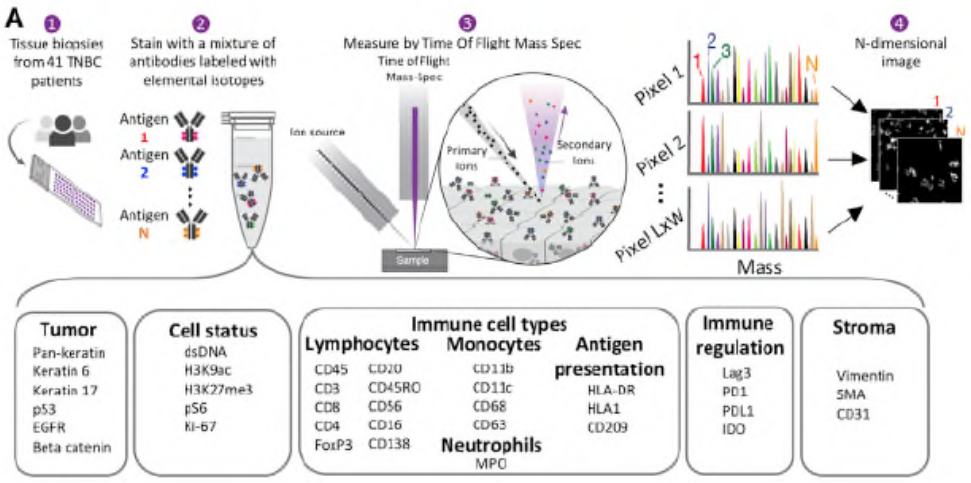
performance of its MIBI technology and improper use of the Fluidigm antibodies and related reagents.

75. The 2018 Press Release claimed that the MIBI technology was invented in Dr. Nolan's lab and subsequently developed by Drs. Bendall and Angelo. The 2018 Press Release reveals that IONpath's MIBI technology "uses secondary-ion mass spectrometry (SIMS), . . . to image antibodies tagged with monoisotopic metal reporters, permitting 40+ proteins to be simultaneously measured."

76. The 2018 Press Release also asserted that IONpath was piloting its "MIBIscope" with "research institutes and biopharmas . . . in which high-fidelity multiplexed imaging data is needed."

77. The IONpath *Cell* publication, titled "A Structured Tumor-Immune Microenvironment in Triple Negative Breach Cancer Revealed by Multiplexed Ion Beam Imaging" (the "2018 Article"), used "multiplexed ion beam imaging by time-of-flight (MIBI-TOF)" to quantify in position expressions of 30+ proteins from certain tissues.

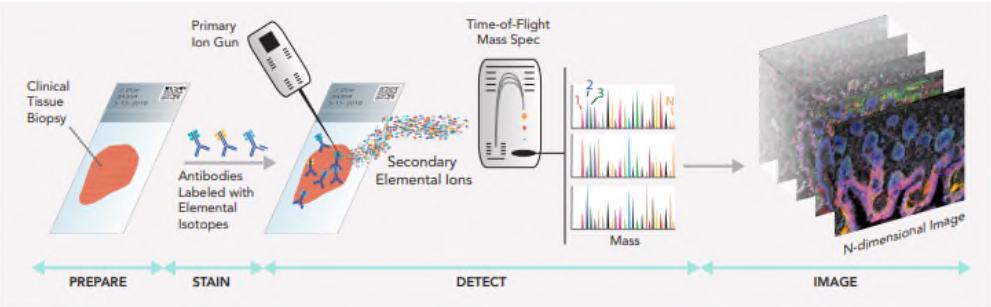
78. The 2018 Article describes the method by which the authors conducted the study; namely, taking tissue biopsies and staining them with "a mixture of antibodies labeled with elemental isotopes." Once stained, the authors' methodology called for raster-scanning the tissues with an ion beam resulting in the generation of secondary elemental ions, which the authors proceeded to analyze doing time-of-flight mass spectrometry.



See 2018 Article at Figure A1 describing MIBI-TOF workflow.

79. The 2018 Article explicitly references certain authors' association with IONpath: "M.A. and S.C.B. [Dr. Bendall] have patents relating to MIBI technology and are board members, shareholders, and consultants in Ionpath Inc."

80. At or about the time the 2018 Article published, IONpath posted on its website a brochure for its MIBIscope I, as discussed above, describing how it works in much the same manner. The brochure advertises that "[t]he MIBIscope system uses Secondary Ion Mass Spectrometry (SIMS) and enables simultaneous imaging of 40+ markers" where "tissue samples are raster scanned with a particle ion beam" and "detected via TOF mass spectrometry."



81. The brochure also included images that, on information and belief, IONpath generated with its MIBIscope using Fluidigm's Maxpar antibodies and related reagents in further breach of the Ts&Cs.

82. IONpath's website further advertises "MIBI™ Reagents for HIGH Multiplex Imaging" and has distributed a brochure describing "isotopically conjugated antibodies" and antibody conjugation kits using antibodies conjugated to lanthanides. IONpath's Safety Data Sheets for MIBItag Conjugation Kits, available on the IONpath website, describe these materials as including lanthanides and polymer components.

83. IONpath's website also advertises its Pharma Partnership service - an in-house analysis method for customers where IONpath conducts "tissue staining, imaging and scanning."

84. On September 18, 2018, IONpath announced that it planned to launch a commercial version of its infringing technology in 2019.

85. Even though previously warned in February 2018, IONpath's blatant breach of the Ts&Cs and encouragement to other Fluidigm customers to engage in the same wrongful conduct resulted in Fluidigm sending another letter to IONpath, on September 24, 2018, seeking IONpath's commitment to refrain from improper and tortious conduct.

86. In addition, in Fluidigm's September 2018 letter, Fluidigm gave notice to IONpath of Fluidigm's recently issued '104 Patent, of which IONpath confirmed it was aware.

V. IONPATH'S IMPROPER AND INFRINGING COMMERCIAL ACTIVITY

87. In February 2019, Fluidigm learned that IONpath had contacted at least one of Fluidigm's customers, on information and belief, in an effort to demonstrate IONpath's MIBIscope and reagents and to sell the infringing technology.

88. On information and belief, by August 2019, IONpath has sold at least six, and possibly more, of its infringing MIBIscopes (and potentially reagents) to various institutions. IONpath has also commercially marketed the MIBItags labeling reagents on its website and brochures.

89. Fluidigm has been harmed by IONpath's flagrant unauthorized use and infringement of Fluidigm's patented methods and technologies.

COUNT I

INTENTIONAL INTERFERENCE WITH CONTRACTUAL RELATIONS

90. Fluidigm incorporates by reference and realleges the averments set forth in the preceding paragraphs as if fully restated and incorporated herein.

91. Fluidigm requires that its customers of the Maxpar® antibodies agree to certain Ts&Cs. The Ts&Cs contain provisions through which Fluidigm's customers agree to not "use any Fluidigm consumables or reagents with any non-Fluidigm instrument, device or system." Ts&Cs at § 3.2.

92. Upon information and belief, IONpath had received, in whole or in part, Fluidigm's Ts&Cs no later than February 2018 and knew that Fluidigm's customers could not "use any Fluidigm consumables or reagents with any non-Fluidigm instrument, device or system." Moreover, in February 2018, Fluidigm gave IONpath notice that using (or encouraging others to use) Fluidigm's antibodies and reagents with a non-Fluidigm system violated the Ts&Cs.

93. In complete disregard for Fluidigm's contracts with its customers and the Ts&Cs, IONpath continued to encourage Fluidigm's customers to breach the Ts&Cs by promoting the use of Fluidigm's antibodies and related reagents with IONpath's technology through, among other means: publications, brochures, press releases, and direct contact.

94. IONpath actions, especially after receiving notice from Fluidigm, were intentional with IONpath's full knowledge that it would result in Fluidigm's customers breaching their contracts with Fluidigm.

95. IONpath's intentional interference with Fluidigm's contracts with its customers has harmed Fluidigm by encouraging unauthorized use of Fluidigm's Maxpar antibodies and related reagents in manners that breach the Ts&Cs and result in unsafe and untested results and uses.

96. IONpath's intentional interference with the Ts&Cs is a substantial factor in causing Fluidigm's loss of business opportunities.

COUNT II
DIRECT PATENT INFRINGEMENT
(The '386 Patent)

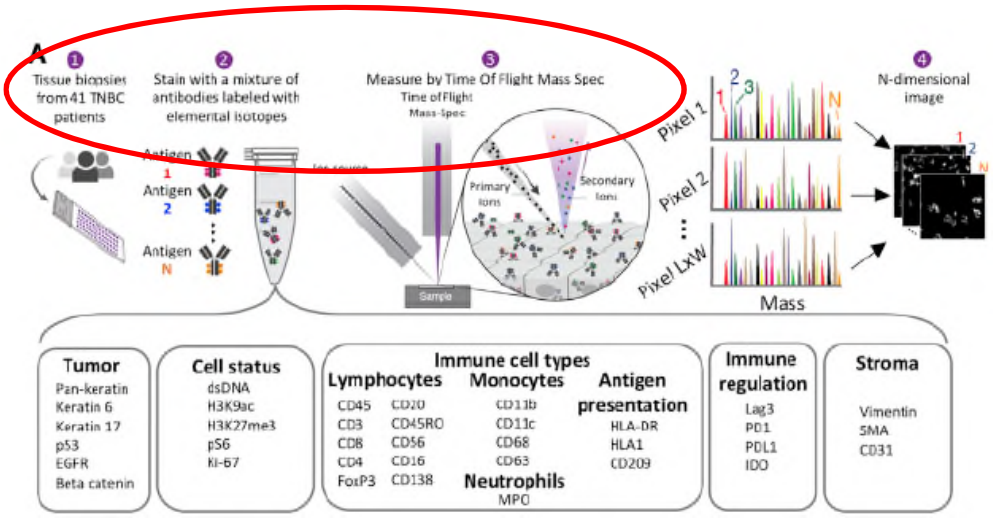
97. Fluidigm incorporates by reference and realleges the averments set forth in the preceding paragraphs as if fully restated and incorporated herein.

98. On January 15, 2019, the Patent Office duly and legally issued the '386 Patent.

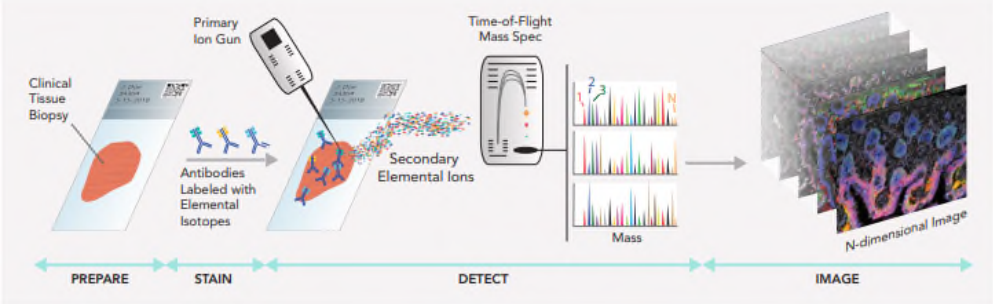
99. Fluidigm is the owner, by assignment, of all right, title, and interest in and to the '386 Patent, including, but not limited to, the right to recover damages for past and future infringement.

100. IONpath has been and is directly infringing, literally and/or under the doctrine of equivalents, the '386 Patent in violation of 35 U.S.C. § 271 by, among other things, using systems and methods for the analysis of a single cell, including, but not limited to, staining by a mixture of antibodies tagged with elemental tags of lanthanide or noble metal, ionizing, and then analyzing using mass spectroscopy and time of flight analysis to detect the signal of the ionized elemental tags, which methods are covered by one or more of the claims of the '386 Patent, including, but not limited to, claim 1.

101. As IONpath published in the 2018 Article, IONpath constructed a "purpose-built instrument" that implements "a method that uses secondary ion mass spectrometry to image antibodies tagged with isotopically pure elemental metal reporters in intact tissue sections" combined with "time-of-flight mass spectrometry . . . ." This is further evidenced by, among other things, the summary IONpath included in the 2018 Article at Figure 1A:



102. IONpath's website also confirms in a brochure that its MIBIscope I is the "purpose-built" machine that implements Fluidigm's patented method:



103. IONpath has also commercialized MIBItags that are isotopically labelled antibodies that implement Fluidigm's patented method when combined with IONpath's MIBIscope. IONpath has used and advocated use of these MIBItags in combination with their IONpath instruments on their website, and has published results obtained with the MIBIscope and identifying MIBItags as the labeling reagents (in the 2018 Article referred to above, *see* Supplementary Material, page e1, Critical Commercial Assays).

STAR★METHODS

KEY RESOURCES TABLE

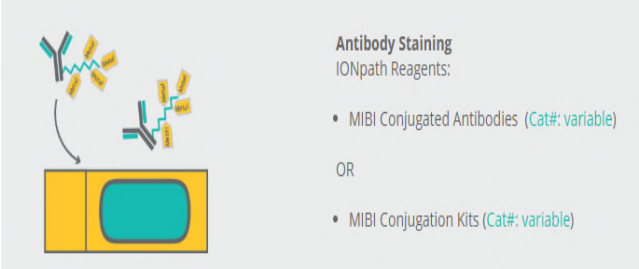
Critical Commercial Assays		
Maxpar X8 Antibody labeling kit	Fluidigm	Cat#2011XXX
MIBItag Conjugation Kit	IONpath	Cat#600XXX
ImmPRESS UNIVERSAL (Anti-Mouse/Anti-Rabbit) IgG KIT (HRP)	Vector Laboratories	Cat#MP-7500-15
ImmPACT DAB (For HRP Substrate)	Vector Laboratories	Cat#SK-4105

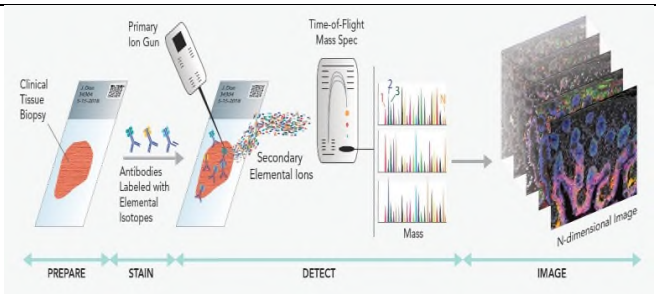
104. On information and belief, at least with respect to the subject matter of the methods disclosed and claimed in the '386 Patent, the use of the "purpose-built" MIBIscope for performing mass spectrometry analysis directly infringes at least claim 1 of the '386 Patent.

105. A chart setting out the elements of claim 1 of the '386 Patent, and certain, but not all, representative corresponding infringing activities of IONpath, is below.

'386 Patent

Claim Element	IONpath Activities
1. A method of sequentially analyzing single cells by mass spectrometry, comprising:	IONpath's **MIBIscope** performs a method of sequentially analyzing single cells by mass spectrometry https://www.ionpath.com/mibi-technology/ MIBI™ technology (Multiplexed Ion Beam Imaging) uses Secondary-Ion Mass Spectrometry (SIMS), a type of mass spectrometry traditionally used in the semiconductor industry, to image antibodies tagged with monoisotopic metal reporters. This unique technology enables: • Visualization of 40+ markers simultaneously • Imaging at the sub-cellular resolution • Detection of low abundance proteins • Rescanning of slides at multiple resolutions https://web.stanford.edu/group/nolan/technologies.html Multiplexed ion beam imaging (MIBI) allows analyzing up to 100 targets simultaneously over a five-log dynamic range in **a way similar to CyTOF**, but in addition to **measuring protein levels on individual cells**, it also provides the information about cell morphology and localization.
providing a sample containing a plurality of tagged cells tagged with a plurality of tagged antibodies, wherein each of the tagged antibodies is specific for a different analyte, and wherein each of the tagged antibodies is tagged with an elemental tag comprising a lanthanide or noble metal;	IONPath's **MIBItags** are used to create a sample containing a plurality of tagged cells tagged with a plurality of tagged antibodies, wherein each of the tagged antibodies is specific for a different analyte, and wherein each of the tagged antibodies is tagged with an elemental tag comprising a lanthanide or noble metal https://www.ionpath.com/mibi-technology/ STAIN: Tissue is stained with a mixture of validated **antibodies** with conjugated **elemental reporters**, in one single step. This unique technology enables: • Visualization of 40+ markers simultaneously • Imaging at the sub-cellular resolution **2018 P106 Poster** Samples were stained with a panel of 15 antibodies, each labeled with a specific metal isotope. (https://www.ionpath.com/wp-

	content/uploads/2018/11/P106_IONpoth_Ptacek_SITC-2018-2.pdf) https://www.ionpath.com/reagents/  SDS from https://www.ionpath.com/antibody-conjugation-kit/ Product identifier: MIBItag Conjugation Kit (Y) Synonyms: None identified Trade names: None identified Chemical family: Lanthanide component Mixture - contains nitric acid and metal lanthanide Stabilization Buffer component Mixture – contains sodium azide Polymer component – contains diethylenetriaminepentaacetic acid **2018 Cell Publication** *(https://doi.org/10.1016/j.cell.2018.08.039)* STAR★METHODS KEY RESOURCES TABLE Critical Commercial Assays Maxpar X8 Antibody labeling kit — Fluidigm — Cat#2011XXX MIBItag Conjugation Kit — IONpath — Cat#600XXX ImmPRESS UNIVERSAL (Anti-Mouse/Anti-Rabbit) IgG KIT (HRP) — Vector Laboratories — Cat#MP-7500-15 ImmPACT DAB (For HRP Substrate) — Vector Laboratories — Cat#SK-4105
vaporizing, atomizing, and ionizing multiple elemental tags from a single first cell of the plurality of tagged cells;	IONpath's **MIBIscope** performs steps of vaporizing, atomizing, and ionizing multiple elemental tags from a single first cell of the plurality of tagged cells https://www.ionpath.com/mibi-technology/

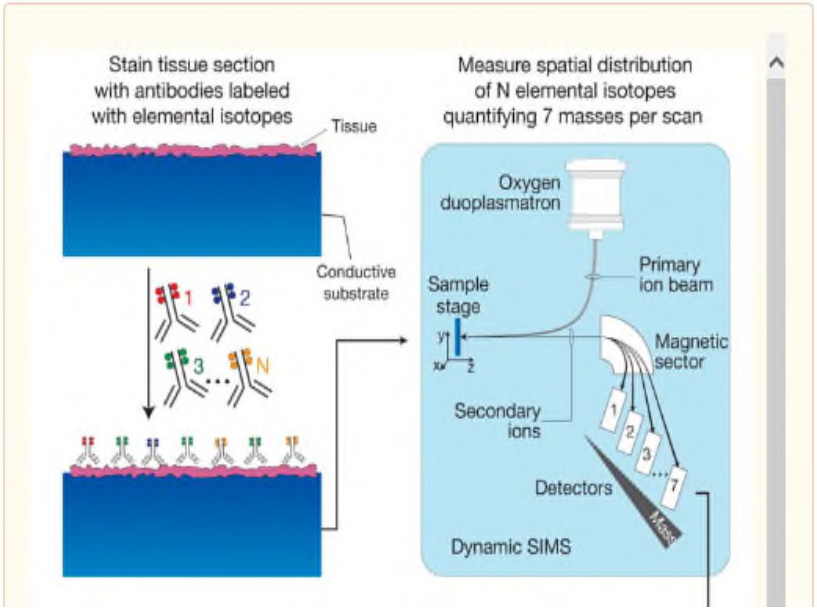


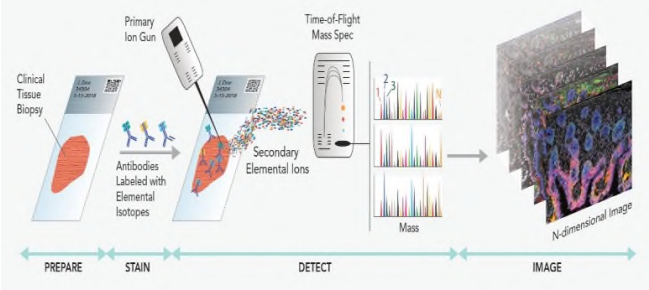
See also 2014 article with Nolan et al. on MIBI technique: (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4110905/):

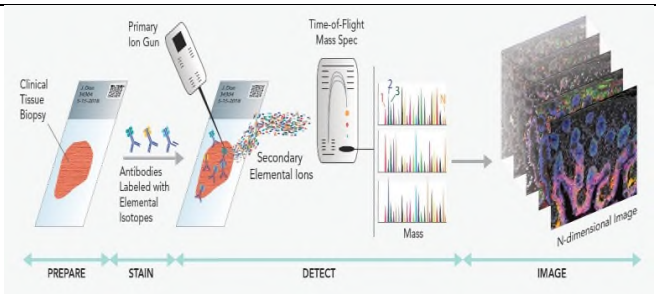
Results Go to:

Performance assessment of MIBI

The workflow for MIBI is comparable to IF and IHC assays (Fig. 1). Instead of fluorophores or enzyme-conjugated reagents, biological specimens are incubated with primary antibodies coupled to stable lanthanides highly enriched for a single isotope (Fig. 1). Primary antibodies are combined in solution for simultaneous incubation with the specimen. The specimens prepared for MIBI are mounted in a sample holder and subjected to a rasterized oxygen duoplasmatron primary ion beam. As this ion beam strikes the sample lanthanide adducts of the bound antibodies are liberated as secondary ions. In this study, the secondary ions are subsequently analyzed via a magnetic sector mass spectrometer equipped with multiple detectors, permitting parallel detection of multiple lanthanide isotopes (mass-based reporters). The resultant data produces a two-dimensional map of the elemental distribution of each lanthanide, and thus each antibody and its corresponding epitope.



detecting, using mass spectrometry, the elemental composition of the first cell by detecting a transient signal of the multiple vaporized, atomized, and ionized elemental tags of the first cell;	IONpath's **MIBIscope** performs steps of detecting, using mass spectrometry, the elemental composition of the first cell by detecting a transient signal of the multiple vaporized, atomized, and ionized elemental tags of the first cell https://www.ionpath.com/mibi-technology/ This unique technology enables: • Visualization of 40+ markers simultaneously • Imaging at the sub-cellular resolution  **2018 Cell Publication** *(https://doi.org/10.1016/j.cell.2018.08.039)* **We have since constructed a purpose-built instrument** that utilizes high brightness primary ion sources, novel ion extraction optics, and **time-of-flight mass spectrometry (TOF**) to increase channel multiplexing and decrease acquisition times 50-fold
vaporizing, atomizing, and ionizing multiple elemental tags from a single second cell of the plurality of tagged cells; and	IONpath's **MIBIscope** performs steps of vaporizing, atomizing, and ionizing multiple elemental tags from a single second cell of the plurality of tagged cells https://www.ionpath.com/mibi-technology/



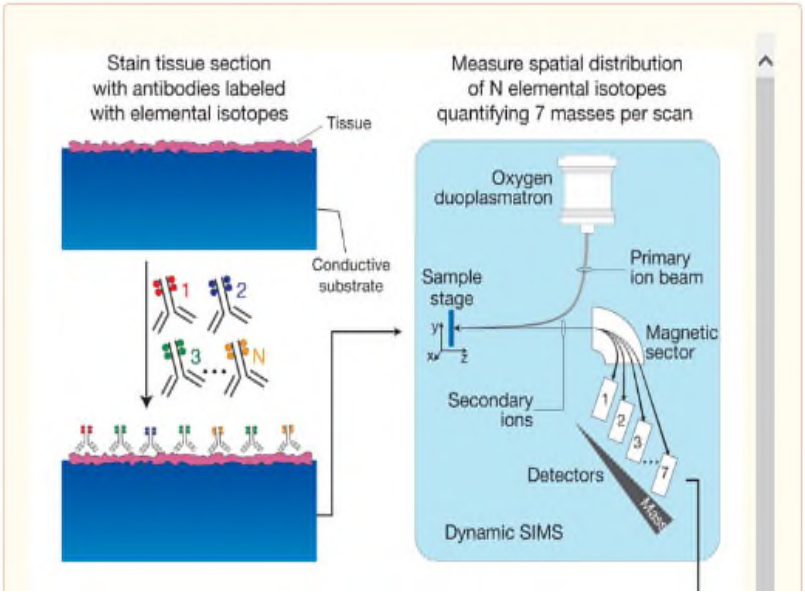
See also 2014 article with Nolan et al. on MIBI technique: (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4110905/):

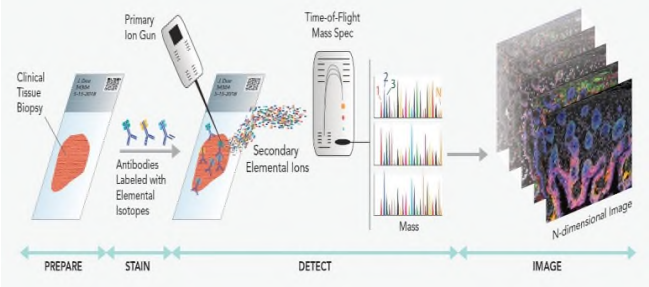
Results

Go to:

Performance assessment of MIBI

The workflow for MIBI is comparable to IF and IHC assays (Fig. 1). Instead of fluorophores or enzyme-conjugated reagents, biological specimens are incubated with primary antibodies coupled to stable lanthanides highly enriched for a single isotope (Fig. 1). Primary antibodies are combined in solution for simultaneous incubation with the specimen. The specimens prepared for MIBI are mounted in a sample holder and subjected to a rasterized oxygen duoplasmatron primary ion beam. As this ion beam strikes the sample lanthanide adducts of the bound antibodies are liberated as secondary ions. In this study, the secondary ions are subsequently analyzed via a magnetic sector mass spectrometer equipped with multiple detectors, permitting parallel detection of multiple lanthanide isotopes (mass-based reporters). The resultant data produces a two-dimensional map of the elemental distribution of each lanthanide, and thus each antibody and its corresponding epitope.



detecting, using mass spectrometry, the elemental composition of the second cell by detecting a transient signal of the multiple vaporized, atomized, and ionized elemental tags of the second cell, wherein the transient signal associated with the first cell and the transient signal associated with the second cell are detected sequentially.	IONpath's **MIBIscope** performs steps of detecting, using mass spectrometry, the elemental composition of the second cell by detecting a transient signal of the multiple vaporized, atomized, and ionized elemental tags of the second cell, wherein the transient signal associated with the first cell and the transient signal associated with the second cell are detected sequentially https://www.ionpath.com/mibi-technology/ This unique technology enables: • Visualization of 40+ markers simultaneously • Imaging at the sub-cellular resolution  **2018 Cell Publication** *(https://doi.org/10.1016/j.cell.2018.08.039)* **We have since constructed a purpose-built instrument** that utilizes high brightness primary ion sources, novel ion extraction optics, and **time-of-flight mass spectrometry (TOF)** to increase channel multiplexing and decrease acquisition times 50-fold

106. At all relevant times, on information and belief, IONpath's acts of infringement of the '386 Patent have been committed and are being committed willfully with full knowledge and

notice of Fluidigm's rights in and to the '386 Patent including, but not limited to, the claims set forth therein.

107. As a direct and proximate result of IONpath's infringement of the '386 Patent, Fluidigm has suffered and continues to suffer damage. Fluidigm is entitled to recover from IONpath Fluidigm's lost profits, and no less than a reasonable royalty, in an amount to be determined at trial.

108. As a direct and proximate result of IONpath's acts of infringement, Fluidigm has been irreparably harmed and will continue to be harmed unless and until IONpath's infringing acts are enjoined and restrained by order of this Court.

COUNT III
INDIRECT PATENT INFRINGEMENT
(The '386 Patent)

109. Fluidigm incorporates by reference and realleges the averments set forth in the preceding paragraphs as if fully restated and incorporated herein.

110. IONpath has induced and continues to induce infringement of one or more claims of the '386 Patent under 35 U.S.C. § 271(b).

111. As discussed above, IONpath commercialized a "purpose-built" MIBIscope that requires users to implement "a method that uses secondary ion mass spectrometry to image antibodies tagged with isotopically pure elemental metal reporters in intact tissue sections" combined with "time-of-flight mass spectrometry," which results in the MIBIscope users infringing upon at least claim 1 of the '386 Patent.

112. IONpath also offers for sale the MIBItag reagents which provide isotopically labelled antibodies for use with the MIBIscope, and/or has encouraged the use of Fluidigm's

proprietary Maxpar® Reagents with the MIBIscope. IONpath has published results obtained with the MIBIscope and identifying both the Maxpar® Reagents and MIBItags as the labeling reagents (in the 2018 Article referred to above, *see* Supplementary Material, page e1, Critical Commercial Assays).

STAR★METHODS

KEY RESOURCES TABLE

Critical Commercial Assays		
Maxpar X8 Antibody labeling kit	Fluidigm	Cat#2011XXX
MIBItag Conjugation Kit	IONpath	Cat#600XXX
ImmPRESS UNIVERSAL (Anti-Mouse/Anti-Rabbit) IgG KIT (HRP)	Vector Laboratories	Cat#MP-7500-15
ImmPACT DAB (For HRP Substrate)	Vector Laboratories	Cat#SK-4105

113. In addition to directly infringing at least one claim of the '386 Patent including, but not limited to, claim 1, IONpath indirectly infringes at least claim 1 of the '386 Patent by instructing, directing, and/or requiring others, including customers, purchasers, users, and developers, to perform the method claimed in at least claim 1, either literally or under the doctrine of equivalents, through the sale of the MIBIscope, and MIBItags, where the claimed methods are performed by either IONpath or its customers, purchasers, users, and developers, or some combination thereof. IONpath knew and/or was willfully blind to the fact that it was inducing others, including customers, purchasers, users, and developers, to infringe one or more claims of the '386 Patent by practicing, either themselves or in conjunction with the IONpath, one or more method claims of the '386 Patent, through sales of the MIBIscope.

114. IONpath knowingly and actively aided and abetted the direct infringement of the '386 Patent by instructing and encouraging its customers, purchasers, users, and developers to use its MIBIscope product and its MIBItag isotopically labelled antibodies. Such instructions and encouragement included, but are not limited to, advising third parties to use the MIBIscope and

MIBItag isotopically labelled antibodies, in an infringing manner, providing a mechanism through which third parties may infringe the '386 Patent, advertising and promoting the use of MIBIscope product and MIBItags in an infringing manner, and distributing guidelines and instructions to third parties on how to use MIBIscope and MIBItags in an infringing manner. For example, IONpath's 2018 brochure "MIBIscope™ I: Multiplexed Tissue Imaging that Transforms Discovery" advertises the benefits and advantages of the MIBIscope for performing mass cytometry methods, and describes how it may be used. IONpath's 2019 "MIBI™ Reagents for High Multiplex Tissue Imaging (Get Results You Can Trust)" advertises the benefits and advantages of IONpath's isotopically conjugated antibodies (MIBItags), and described how they can be used for mass cytometry methods. IONpath's website also provides general instructions and explanations for the use of the MIBIscope and MIBItags, along with contact information for obtaining further information and/or ordering these products.

115. As a direct and proximate result of IONpath's infringement of the '386 Patent, Fluidigm has suffered and continues to suffer damage. Fluidigm is entitled to recover from IONpath Fluidigm's lost profits, and no less than a reasonable royalty, in an amount to be determined at trial.

116. As a direct and proximate result of IONpath's acts of infringement, Fluidigm has been irreparably harmed and will continue to be harmed unless and until IONpath's infringing acts are enjoined and restrained by order of this Court.

COUNT IV
CONTRIBUTORY PATENT INFRINGEMENT
(The '386 Patent)

117. Fluidigm incorporates by reference and realleges the averments set forth in the preceding paragraphs as if fully recited and incorporated herein.

118. IONpath has contributed and continues to contribute to the infringement of one or more claims of the '386 Patent under 35 U.S.C. § 271(c).

119. IONpath commercialized a "purpose-built" MIBIscope that implements a method patented by Fluidigm "that uses secondary ion mass spectrometry to image antibodies tagged with isotopically pure elemental metal reporters in intact tissue sections" combined with "time-of-flight mass spectrometry." When analyzing tissue cells with the MIBIscope combined with antibodies tagged with lanthanide or noble metal tags, the MIBIscope user infringes upon a method covered under one or more claims of the '386 Patent including, but not limited to, as set forth in, for example, claim 1 of the '386 Patent.

120. On information and belief, beginning in 2018 or earlier, with an expected commercial launch date in 2019, IONpath knowingly, intentionally, and willfully began marketing and selling the infringing MIBIscope machine. The MIBIscope is a physical apparatus designed to implement Fluidigm's patented method.

121. On information and belief, the MIBIscope is a "purpose-built" machine that does not serve any non-infringing use, but is designed to practice the method of at least Claim 1 of the '386 patent.

122. IONpath has commercialized MIBItags which are isotopically labelled antibodies that implement the method of at least Claim 1 of the '386 patent. When analyzing tissue cells with the MIBItags combined with mass cytometry using the MIBIscope, the MIBItag user infringes upon a patented method as set forth in, for example, claim 1 of the '386 Patent.

123. On information and belief, beginning at least in 2019, IONpath began marketing and selling the infringing MIBItags. The MIBItags are isotopically labelled antibodies designed to implement Fluidigm's patented method.

124. On information and belief, the MIBItags do not serve any non-infringing use.

125. As a direct and proximate result of IONpath's infringement of the '386 Patent, Fluidigm has suffered and continues to suffer damage. Fluidigm is entitled to recover from IONpath Fluidigm's lost profits, and no less than a reasonable royalty, in an amount to be determined at trial.

126. As a direct and proximate result of IONpath's acts of infringement, Fluidigm has been irreparably harmed and will continue to be harmed unless and until IONpath's infringing acts are enjoined and restrained by order of this Court.

COUNT V
DIRECT PATENT INFRINGEMENT
(The '104 Patent)

127. Fluidigm incorporates by reference and realleges the averments set forth in the preceding paragraphs as if fully recited and incorporated herein.

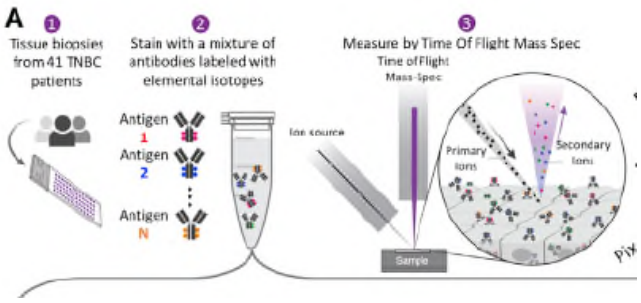
128. On September 11, 2018, the U.S. Patent and Trademark Office duly and legally issued the '104 Patent.

129. Fluidigm is the owner, by assignment and through its wholly owned subsidiary, of all right, title, and interest in and to the '104 Patent, including, but not limited to, the right to recover damages for past and future infringement.

130. IONpath has been and is directly infringing, literally and/or under the doctrine of equivalents, the '104 Patent in violation of 35 U.S.C. § 271 by, among other things, using systems and methods for the analysis of a single cell including, but not limited to, staining by a mixture of antibodies tagged with elemental tags of lanthanide or noble metal, ionizing, and then analyzing using mass spectroscopy and time of flight analysis to detect the signal of the ionized elemental tags, which methods are covered by one or more of the claims of the '104 Patent, including, but not limited to, claim 1.

131. On information and belief, at least with respect to the subject matter of the methods described and claimed in the '104 Patent, the use of the MIBIscope for performing mass spectrometry analysis directly infringes at least claim 1 of the '104 Patent.

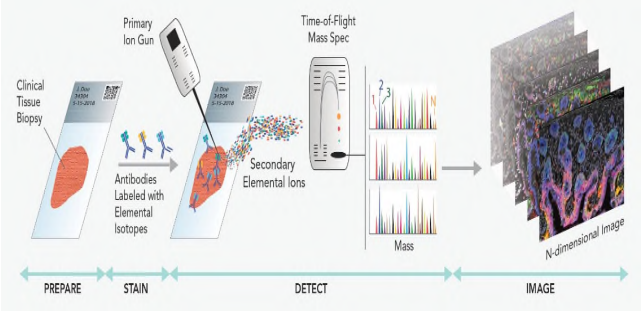
132. As IONpath states in its 2018 Article, IONpath's apparatus infringes on Fluidigm's '104 Patent, Claim 1, by implementing a method wherein IONpath analyzes "specimens . . . placed on a slide and stained overnight using a single mater mix of elementally labeled primary antibodies." The slide is then "placed in the MIBI-TOF mass spectrometer" for analysis. IONpath's description of the method used by its apparatus also confirms that the analysis does not require prior acidification of the sample. This is summarized in Figure 1A of the 2018 Article:

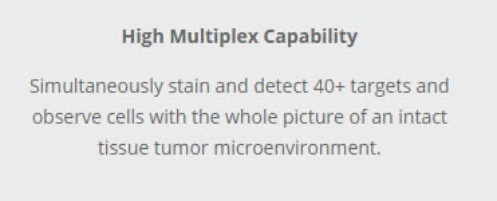
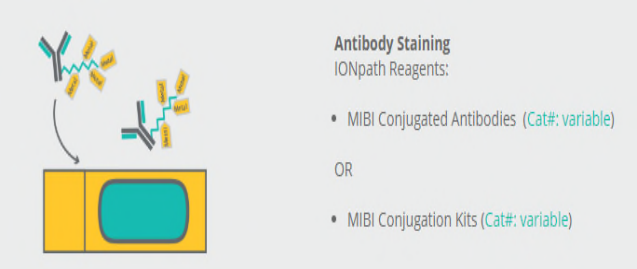


133. IONpath has also used and commercialized MIBItags that are isotopically labelled antibodies that implement Fluidigm's patented method when combined with IONPath's MIBIscope.

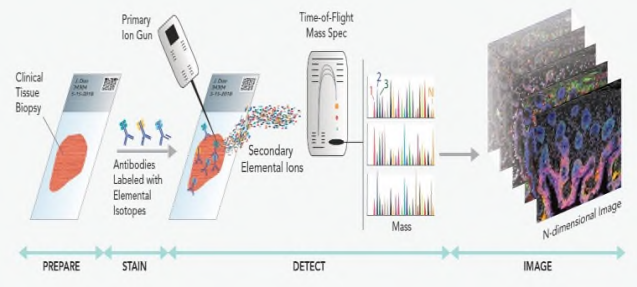
134. A chart setting out the elements of claim 1 of the '104 Patent, and the corresponding infringing activities of IONpath, is below.

'104 Patent

Claim Limitation	**IONpath/customer activities**
1. A method for the analysis of an analyte in a sample, comprising:	IONpath's **MIBIscope** performs a method for the analysis of an analyte in a sample https://www.ionpath.com/mibi-technology/  https://www.ionpath.com/

	 https://web.stanford.edu/group/nolan/technologies.html Multiplexed ion beam imaging (MIBI) allows analyzing up to 100 targets simultaneously over a five-log dynamic range in **a way similar to CyTOF**, but in addition to **measuring protein levels on individual cells**, it also provides the information about cell morphology and localization.
(i) incubating an element tagged affinity reagent with an analyte, the element tagged affinity reagent comprising an affinity reagent tagged with an element tag,	IONpath's **MIBItags** are used for incubating an element tagged affinity reagent with an analyte, the element tagged affinity reagent comprising an affinity reagent tagged with an element tag https://www.ionpath.com/mibi-technology/ STAIN: Tissue is stained with a mixture of validated **antibodies** with conjugated **elemental reporters**, in one single step. https://www.ionpath.com/reagents/ 
the element tag comprising a linear or branched polymer having multiple metal-binding pendant groups, wherein each pendant group includes at	IONpath's **MIBItags** are used for providing the element tag comprising a linear or branched polymer having multiple metal-binding pendant groups, wherein each pendant group includes at least one metal atom or is capable of binding at least one metal atom

least one metal atom or is capable of binding at least one metal atom,	SDS from https://www.ionpath.com/antibody-conjugation-kit/ **Product identifier** MIBItag Conjugation Kit (Y) **Synonyms** None identified **Trade names** None identified **Chemical family** Lanthanide component Mixture - contains nitric acid and metal lanthanide Stabilization Buffer component Mixture – contains sodium azide Polymer component – contains diethylenetriaminepentaacetic acid **2018 Cell Publication** *(https://doi.org/10.1016/j.cell.2018.08.039)* **STAR★METHODS** **KEY RESOURCES TABLE** Critical Commercial Assays Maxpar X8 Antibody labeling kit — Fluidigm — Cat#2011XXX MIBItag Conjugation Kit — IONpath — Cat#600XXX ImmPRESS UNIVERSAL (Anti-Mouse/Anti-Rabbit) IgG KIT (HRP) — Vector Laboratories — Cat#MP-7500-15 ImmPACT DAB (For HRP Substrate) — Vector Laboratories — Cat#SK-4105
and wherein the affinity reagent specifically binds with the analyte, wherein the analyte is located within or on an intact cell;	IONpath's **MIBItags** are used to provide an affinity reagent that specifically binds with the analyte, wherein the analyte is located within or on an intact cell https://www.ionpath.com/ **FFPE Tissue Staining** Follow a classic multiplexed IHC protocol using IONpath pathologist-validated or custom antibodies with any FFPE samples. **High Multiplex Capability** Simultaneously stain and detect 40+ targets and observe cells with the whole picture of an intact tissue tumor microenvironment.
(ii) separating unbound element tagged affinity reagent from bound element tagged affinity reagent; and	IONpath's use of the **MIBItags** involves separating unbound element tagged affinity reagent from bound element tagged affinity reagent https://www.ionpath.com/slides-and-reagents/

	MIBI 20X TBS-T Catalog: 567005 Storage: Store at 4°C Description Tris buffered saline (TBS), pH 7.6, with the detergent Tween® 20 is intended to be used as a wash buffer to remove nonspecific antibody binding and as a blocking buffer diluent in the MIBI Staining Protocol.
(iii) analyzing the element tag bound to the affinity reagent attached to the analyte of the intact cell by atomic spectroscopy, wherein analyzing occurs without prior acidification of the sample.	IONpath's **MIBIscope** performs steps of analyzing the element tag bound to the affinity reagent attached to the analyte of the intact cell by atomic spectroscopy, wherein analyzing occurs without prior acidification of the sample https://www.ionpath.com/ **High Multiplex Capability** Simultaneously stain and detect 40+ targets and observe cells with the whole picture of an intact tissue tumor microenvironment. https://www.ionpath.com/mibi-technology/ MIBI™ technology (Multiplexed Ion Beam Imaging) uses Secondary-Ion Mass Spectrometry (SIMS), a type of mass spectrometry traditionally used in the semiconductor industry, to image antibodies tagged with monoisotopic metal reporters. IMAGE: All markers are imaged at the same time and detected via TOF mass spectrometry. 

135. At all relevant times, on information and belief, IONpath's acts of infringement of the '104 Patent have been committed and are being committed with full knowledge of Fluidigm's rights in the '104 Patent.

136. As a direct and proximate result of IONpath's infringement of the '104 Patent, Fluidigm has suffered and continues to suffer damage. Fluidigm is entitled to recover from IONpath Fluidigm's lost profits, but no less than a reasonable royalty, in an amount to be determined at trial.

137. As a direct and proximate result of IONpath's acts of infringement, Fluidigm has been irreparably harmed and will continue to be harmed unless and until IONpath's infringing acts are enjoined and restrained by order of this Court.

COUNT VI
INDIRECT PATENT INFRINGEMENT
(The '104 Patent)

138. Fluidigm incorporates by reference and realleges the averments set forth in the preceding paragraphs as if fully restated and incorporated herein.

139. IONpath has induced and continues to induce infringement of one or more claims of the '104 Patent under 35 U.S.C. § 271(b).

140. In addition to directly infringing at least one claim of the '104 Patent, including, but not limited to, claim 1, IONpath indirectly infringes at least claim 1 of the '104 Patent by instructing, directing, and/or requiring others, including customers, purchasers, users, and

developers, to perform the method claimed in at least claim 1, either literally or under the doctrine of equivalents, through the sale of the MIBIscope (IONpath's MIBI-TOF apparatus), where the claimed methods are performed by either IONpath or its customers, purchasers, users, and developers, or some combination thereof. IONpath knew and/or was willfully blind to the fact that it was inducing others, including customers, purchasers, users, and developers, to infringe one or more claims of the '104 Patent by practicing, either themselves or in conjunction with the IONpath, one or more method claims of the '104 Patent, through sales of the MIBIscope.

141. IONpath knowingly and actively aided and abetted the direct infringement of the '104 Patent by instructing and encouraging its customers, purchasers, users, and developers to use its MIBIscope product. Such instructions and encouragement included, but are not limited to, advising third parties to use MIBIscope in an infringing manner, providing a mechanism through which third parties may infringe the '104 Patent, advertising and promoting the use of MIBIscope product in an infringing manner, and distributing guidelines and instructions to third parties on how to use MIBIscope in an infringing manner. For example, IONpath's 2018 brochure "MIBIscope™ I: Multiplexed Tissue Imaging that Transforms Discovery" from advertises the benefits and advantages of the MIBIscope for performing mass cytometry methods, and describes how it may be used. IONpath's website also provides general instructions and explanations for the use of the MIBIscope, along with contact information for further information on these products.

142. IONpath also knowingly and actively aided and abetted the direct infringement of the '104 Patent by instructing and encouraging its customers, purchasers, users, and developers

to use its MIBItag isotopically conjugated antibodies with its MIBIscope product. Such instructions and encouragement included, but are not limited to, advising third parties to use the MIBItags in an infringing manner, providing a mechanism through which third parties may infringe the '104 Patent, advertising and promoting the use of MIBItags in an infringing manner, and distributing guidelines and instructions to third parties on how to use MIBItags in an infringing manner. For example, IONPath's 2019 "MIBI™ Reagents for High Multiplex Tissue Imaging (Get Results You Can Trust)" advertises the benefits and advantages of IONpath's isotopically conjugated antibodies (MIBItags), and described how they can be used for mass cytometry methods. IONpath's website also provides general instructions and explanations for the use of the MIBItags, along with contact information for obtaining further information and/or ordering these products.

143. As a direct and proximate result of IONpath's infringement of the '104 Patent, Fluidigm has suffered and continues to suffer damage. Fluidigm is entitled to recover from IONpath Fluidigm's lost profits, and no less than a reasonable royalty, in an amount to be determined at trial.

144. As a direct and proximate result of IONpath's acts of infringement, Fluidigm has been irreparably harmed and will continue to be harmed unless and until IONpath's infringing acts are enjoined and restrained by order of this Court.

COUNT VII
CONTRIBUTORY PATENT INFRINGEMENT
(The '104 Patent)

145. Fluidigm incorporates by reference and realleges the averments set forth in the preceding paragraphs as if fully recited and incorporated herein.

146. IONpath has contributed and continues to contribute to the infringement of one or more claims of the '104 Patent under 35 U.S.C. § 271(c).

147. IONpath commercialized its MIBIscope (IONpath's MIBI-TOF apparatus) that implements a method patented by Fluidigm by analyzing antibodies stained with multiple metal tags that will specifically bind with specified analytes located within or on intact cells, by means of spectroscopy without prior acidification of the sample. IONpath has also commercialized its MIBItag isotopically labelled antibodies for use with the patented method. When analyzing tissue cells with the MIBIscope that have been stained with multiple lanthanide or noble metal tags, the MIBIscope user infringes upon a method covered under one or more claims of the '104 Patent including, but not limited to, as set forth in, for example, claim 1 of the '104 Patent. Similarly, when staining cells with the MIBItags and then analyzing tissue cells with the MIBIscope, the MIBItag user infringes upon a patented method as set forth in, for example, claim 1 of the '104 Patent.

148. On information and belief, beginning in 2018 or earlier, with an expected commercial launch date in 2019, IONpath knowingly, intentionally and willfully began marketing and selling the infringing MIBIscope machine. The MIBIscope is a physical

apparatus designed to implement Fluidigm's patented method. IONpath is currently advertising the MIBItags for purchase on its website.

149. On information and belief, the MIBIscope is a "purpose-built" machine that does not serve any non-infringing use, but is designed to practice the method of at least claim 1 of the '104 Patent.

150. IONpath has commercialized the MIBItags which are isotopically labelled antibodies that implement the method of at least claim 1 of the '104 Patent.

151. As a direct and proximate result of IONpath's infringement of the '104 Patent, Fluidigm has suffered and continues to suffer damage. Fluidigm is entitled to recover from IONpath Fluidigm's lost profits, and no less than a reasonable royalty, in an amount to be determined at trial.

152. As a direct and proximate result of IONpath's acts of infringement, Fluidigm has been irreparably harmed and will continue to be harmed unless and until IONpath's infringing acts are enjoined and restrained by order of this Court.

COUNT VIII
DIRECT PATENT INFRINGEMENT
(The '698 Patent)

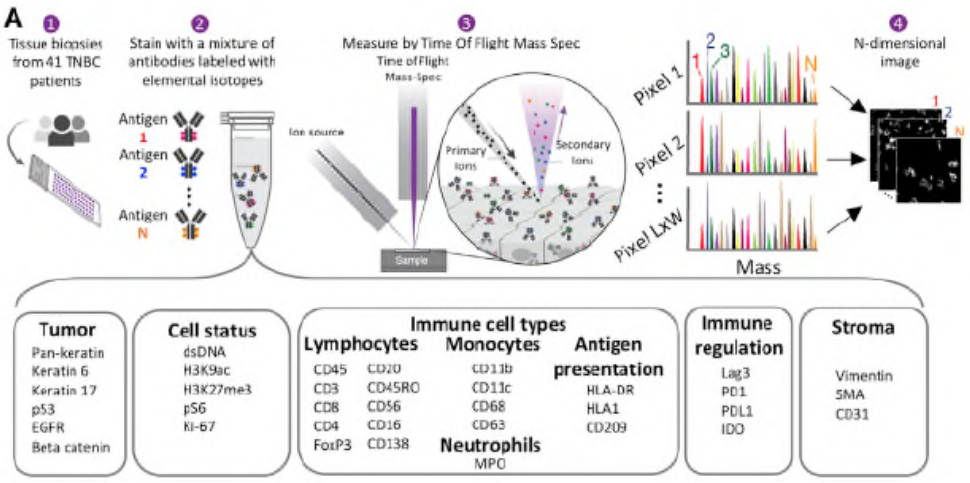
153. Fluidigm incorporates by reference and realleges the averments set forth in the preceding paragraphs as if fully restated and incorporated herein.

154. On October 8, 2019, the Patent Office duly and legally issued the '698 Patent.

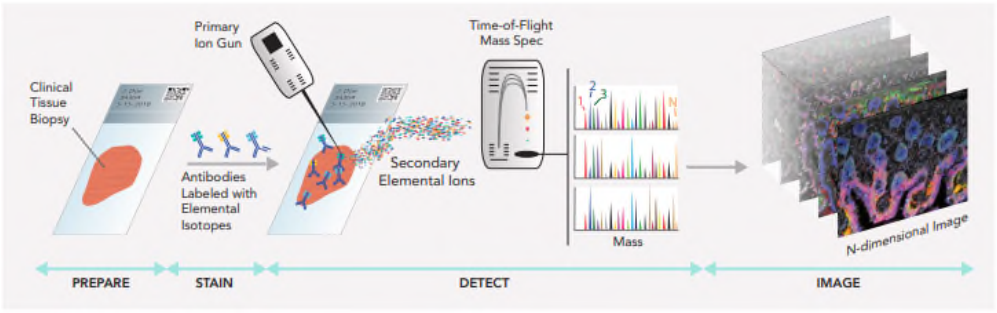
155. Fluidigm is the owner, by assignment, of all right, title, and interest in and to the '698 Patent, including, but not limited to, the right to recover damages for past and future infringement.

156. IONpath has been and is directly infringing, literally and/or under the doctrine of equivalents, the '698 Patent in violation of 35 U.S.C. § 271 by, among other things, making, using, offering for sale, and selling systems for the analysis of a single cell, including, but not limited to, systems devised to ionize and then analyze samples using mass spectroscopy and time of flight analysis to detect the signal of ionized elemental tags, where the samples are stained with a mixture of antibodies tagged with elemental tags of lanthanide or noble metal, which systems are covered by one or more of the claims of the '698 Patent, including, but not limited to, claim 1.

157. As IONpath published in the 2018 Article, IONpath constructed a "purpose-built instrument" that "uses secondary ion mass spectrometry to image antibodies tagged with isotopically pure elemental metal reporters in intact tissue sections" in combination with "time-of-flight mass spectrometry . . . ." With respect to the operation of the instrument, the 2018 Article further describes that "tissue is subjected to a nanometer-scale, rasterizing oxygen duoplasma primary ion beam" and "[a]s this ion beam strikes the sample, elemental reporters conjugated to the antibodies are liberated as secondary ions, which are measured and quantified by a time-of-flight mass spectrometer." This is further evidenced by, among other things, the system set forth in the summary IONpath included in the 2018 Article at Figure 1A:



158. IONpath's website also confirms in a brochure that its MIBIscope I contains the elements of Fluidigm's patented system:



159. IONpath has also commercialized MIBItags that are isotopically labelled antibodies that are designed for use with Fluidigm's patented system. IONpath has used and advocated use of these MIBItags in combination with their infringing IONpath instruments on their website, and has published results obtained with the infringing MIBIscope and identifying MIBItags as the labeling reagents (in the 2018 Article referred to above, *see* Supplementary Material, page e1, Critical Commercial Assays).

STAR★METHODS

KEY RESOURCES TABLE

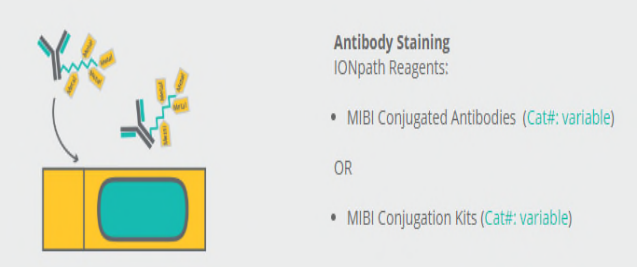
Critical Commercial Assays		
Maxpar X8 Antibody labeling kit	Fluidigm	Cat#2011XXX
MIBItag Conjugation Kit	IONpath	Cat#600XXX
ImmPRESS UNIVERSAL (Anti-Mouse/Anti-Rabbit) IgG KIT (HRP)	Vector Laboratories	Cat#MP-7500-15
ImmPACT DAB (For HRP Substrate)	Vector Laboratories	Cat#SK-4105

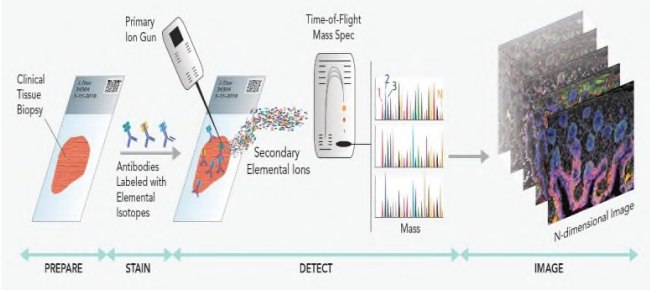
160. On information and belief, at least with respect to the subject matter of the systems disclosed and claimed in the '698 Patent, the making, using, offering for sale, and selling of the MIBIscope for performing mass spectrometry analysis directly infringes at least claim 1 of the '698 Patent.

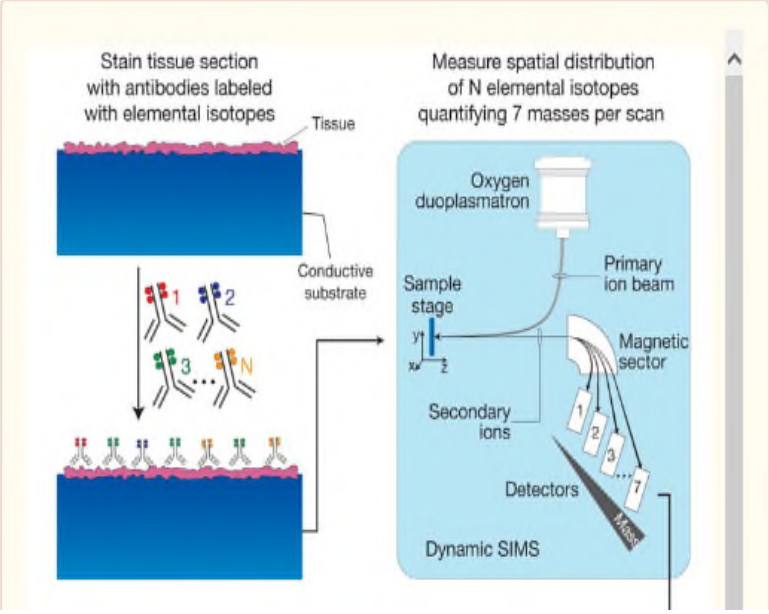
161. A chart setting out the elements of claim 1 of the '698 Patent, and certain, but not all, representative corresponding infringing activities of IONpath, is below.

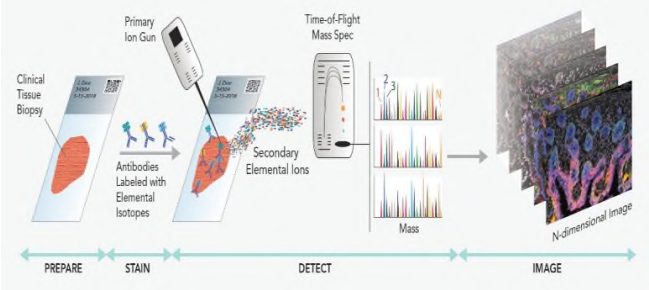
'698 Patent

Claim Element	**IONpath Activities**
1. A system for sequentially analyzing single cells in a sample by mass spectrometry,	IONpath's **MIBIscope** is a system for sequentially analyzing single cells in a sample by mass spectrometry https://www.ionpath.com/mibi-technology/ MIBI™ technology (Multiplexed Ion Beam Imaging) uses Secondary-Ion Mass Spectrometry (SIMS), a type of mass spectrometry traditionally used in the semiconductor industry, to image antibodies tagged with monoisotopic metal reporters. This unique technology enables: • Visualization of 40+ markers simultaneously • Imaging at the sub-cellular resolution • Detection of low abundance proteins • Rescanning of slides at multiple resolutions https://web.stanford.edu/group/nolan/technologies.html Multiplexed ion beam imaging (MIBI) allows analyzing up to 100 targets simultaneously over a five-log dynamic range in **a way similar to CyTOF**, but in addition to **measuring protein levels on individual cells**, it also provides the information about cell morphology and localization.
wherein the sample comprises a plurality of	IONPath's **MIBItags** are used to create a sample comprising a plurality of tagged cells tagged with a plurality of tagged antibodies, wherein each of

tagged cells tagged with a plurality of tagged antibodies, wherein each of the plurality of tagged antibodies is specific for a different analyte, and wherein each of the plurality of tagged antibodies is tagged with an elemental tag comprising a lanthanide or noble metal;	the plurality of tagged antibodies is specific for a different analyte, and wherein each of the plurality of tagged antibodies is tagged with an elemental tag comprising a lanthanide or noble metal https://www.ionpath.com/mibi-technology/ STAIN: Tissue is stained with a mixture of validated **antibodies** with conjugated **elemental reporters**, in one single step. This unique technology enables: • Visualization of 40+ markers simultaneously • Imaging at the sub-cellular resolution **2018 P106 Poster** Samples were stained with a panel of 15 antibodies, each labeled with a specific metal isotope. (https://www.ionpath.com/wp-content/uploads/2018/11/P106_IONpoth_Ptacek_SITC-2018-2.pdf) https://www.ionpath.com/reagents/  SDS from https://www.ionpath.com/antibody-conjugation-kit/ Product identifier: MIBItag Conjugation Kit (Y) Synonyms: None identified Trade names: None identified Chemical family: Lanthanide component Mixture - contains nitric acid and metal lanthanide Stabilization Buffer component Mixture – contains sodium azide Polymer component – contains diethylenetriaminepentaacetic acid **2018 Cell Publication**

	(https://doi.org/10.1016/j.cell.2018.08.039) **STAR★METHODS** **KEY RESOURCES TABLE** Critical Commercial Assays Maxpar X8 Antibody labeling kit — Fluidigm — Cat#2011XXX MIBItag Conjugation Kit — IONpath — Cat#600XXX ImmPRESS UNIVERSAL (Anti-Mouse/Anti-Rabbit) IgG KIT (HRP) — Vector Laboratories — Cat#MP-7500-15 ImmPACT DAB (For HRP Substrate) — Vector Laboratories — Cat#SK-4105
wherein the system comprises: a first device to vaporize, atomize, and ionize multiple elemental tags from a single first cell of the plurality of tagged cells and multiple elemental tags from a single second cell of the plurality of tagged cells;	IONpath's **MIBIscope** is a system having a first device to vaporize, atomize, and ionize multiple elemental tags from a single first cell of the plurality of tagged cells and multiple elemental tags from a single second cell of the plurality of tagged cells https://www.ionpath.com/mibi-technology/  See also 2014 article with Nolan et al. on MIBI technique: (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4110905/):

	Results Go to: Performance assessment of MIBI The workflow for MIBI is comparable to IF and IHC assays (Fig. 1). Instead of fluorophores or enzyme-conjugated reagents, biological specimens are incubated with primary antibodies coupled to stable lanthanides highly enriched for a single isotope (Fig. 1). Primary antibodies are combined in solution for simultaneous incubation with the specimen. The specimens prepared for MIBI are mounted in a sample holder and subjected to a rasterized oxygen duoplasmatron primary ion beam. As this ion beam strikes the sample lanthanide adducts of the bound antibodies are liberated as secondary ions. In this study, the secondary ions are subsequently analyzed via a magnetic sector mass spectrometer equipped with multiple detectors, permitting parallel detection of multiple lanthanide isotopes (mass-based reporters). The resultant data produces a two-dimensional map of the elemental distribution of each lanthanide, and thus each antibody and its corresponding epitope. 
a second device to detect, by mass spectrometry, lanthanides and/or noble metals of the single first cell by detecting a transient signal of the multiple vaporized, atomized, and ionized elemental tags of the single first cell, and lanthanides and/or noble metals of the single second cell by detecting a	IONpath's **MIBIscope** is a system having a second device to detect, by mass spectrometry, lanthanides and/or noble metals of the single first cell by detecting a transient signal of the multiple vaporized, atomized, and ionized elemental tags of the single first cell, and lanthanides and/or noble metals of the single second cell by detecting a transient signal of the multiple vaporized, atomized, and ionized elemental tags of the single second cell, wherein the transient signal associated with the single first cell and the transient signal associated with the single second cell are detected sequentially https://www.ionpath.com/mibi-technology/

transient signal of the multiple vaporized, atomized, and ionized elemental tags of the single second cell, wherein the transient signal associated with the single first cell and the transient signal associated with the single second cell are detected sequentially.	This unique technology enables: • Visualization of 40+ markers simultaneously • Imaging at the sub-cellular resolution  **2018 Cell Publication** *(https://doi.org/10.1016/j.cell.2018.08.039)* **We have since constructed a purpose-built instrument** that utilizes high brightness primary ion sources, novel ion extraction optics, and **time-of-flight mass spectrometry (TOF)** to increase channel multiplexing and decrease acquisition times 50-fold

162. At all relevant times, on information and belief, IONpath's acts of infringement of the '698 Patent have been committed and are being committed willfully with full knowledge and notice of Fluidigm's rights in and to the '698 Patent, and its family, including, but not limited to, the claims set forth in the '698 Patent.

163. As a direct and proximate result of IONpath's infringement of the '698 Patent, Fluidigm has suffered and continues to suffer damage. Fluidigm is entitled to recover from

IONpath Fluidigm's lost profits, and no less than a reasonable royalty, in an amount to be determined at trial.

164. As a direct and proximate result of IONpath's acts of infringement, Fluidigm has been irreparably harmed and will continue to be harmed unless and until IONpath's infringing acts are enjoined and restrained by order of this Court.

COUNT IX
INDIRECT PATENT INFRINGEMENT
(The '698 Patent)

165. Fluidigm incorporates by reference and realleges the averments set forth in the preceding paragraphs as if fully restated and incorporated herein.

166. IONpath has induced and continues to induce infringement of one or more claims of the '698 Patent under 35 U.S.C. § 271(b).

167. As discussed above, IONpath commercialized its MIBIscope instrument that "uses secondary ion mass spectrometry to image antibodies tagged with isotopically pure elemental metal reporters in intact tissue sections" combined with "time-of-flight mass spectrometry," the operation of which results in the MIBIscope users infringing upon at least claim 1 of the '698 Patent.

168. IONpath also offers for sale the MIBItag reagents which can be used to provide the sample including isotopically labelled antibodies for use with its infringing MIBIscope, and/or has encouraged the use of Fluidigm's proprietary Maxpar® Reagents with the MIBIscope. IONpath has published results obtained with the MIBIscope and identifying both the Maxpar® Reagents and MIBItags as the labeling reagents (in the 2018 Article referred to above, *see* Supplementary Material, page e1, Critical Commercial Assays).

STAR★METHODS

KEY RESOURCES TABLE

Critical Commercial Assays		
Maxpar X8 Antibody labeling kit	Fluidigm	Cat#2011XXX
MIBItag Conjugation Kit	IONpath	Cat#600XXX
ImmPRESS UNIVERSAL (Anti-Mouse/Anti-Rabbit) IgG KIT (HRP)	Vector Laboratories	Cat#MP-7500-15
ImmPACT DAB (For HRP Substrate)	Vector Laboratories	Cat#SK-4105

169. In addition to directly infringing at least one claim of the '698 Patent including, but not limited to, claim 1, IONpath indirectly infringes at least claim 1 of the '698 Patent by instructing, directing, and/or requiring others, including customers, purchasers, users, and developers, to use the system claimed in at least claim 1, either literally or under the doctrine of equivalents, through the sale of the MIBIscope, either alone or in combination with the sale of MIBItags, where the claimed systems are operated by either IONpath or its customers, purchasers, users, and developers, or some combination thereof. IONpath knew and/or was willfully blind to the fact that it was inducing others, including customers, purchasers, users, and developers, to infringe one or more claims of the '698 Patent by practicing, either themselves or in conjunction with IONpath, one or more system claims of the '698 Patent, through sales of the MIBIscope.

170. IONpath knowingly and actively aided and abetted the direct infringement of the '698 Patent by instructing and encouraging its customers, purchasers, users, and developers to use its MIBIscope product, including in combination with its MIBItag isotopically labelled antibodies. Such instructions and encouragement included, but are not limited to, advising third parties to use the MIBIscope in an infringing manner, including but not limited to use in combination with the MIBItag isotopically labelled antibodies, providing a mechanism through which third parties may infringe the '698 Patent, advertising and promoting the use of

MIBIscope product alone and in combination with the MIBItags in an infringing manner, and distributing guidelines and instructions to third parties on how to use MIBIscope alone and in combination with the MIBItags in an infringing manner. For example, IONpath's 2018 brochure "MIBIscope™ I: Multiplexed Tissue Imaging that Transforms Discovery" advertises the benefits and advantages of the MIBIscope for performing mass cytometry methods, and describes how it may be used. IONpath's 2019 "MIBI™ Reagents for High Multiplex Tissue Imaging (Get Results You Can Trust)" advertises the benefits and advantages of IONpath's isotopically conjugated antibodies (MIBItags), and described how they can be used for mass cytometry systems. IONpath's website also provides general instructions and explanations for the use of the MIBIscope, and the MIBItags, along with contact information for obtaining further information and/or ordering these products.

171. As a direct and proximate result of IONpath's infringement of the '698 Patent, Fluidigm has suffered and continues to suffer damage. Fluidigm is entitled to recover from IONpath Fluidigm's lost profits, and no less than a reasonable royalty, in an amount to be determined at trial.

172. As a direct and proximate result of IONpath's acts of infringement, Fluidigm has been irreparably harmed and will continue to be harmed unless and until IONpath's infringing acts are enjoined and restrained by order of this Court.

COUNT X
CONTRIBUTORY PATENT INFRINGEMENT
(The '698 Patent)

173. Fluidigm incorporates by reference and realleges the averments set forth in the preceding paragraphs as if fully recited and incorporated herein.

174. IONpath has contributed and continues to contribute to the infringement of one or more claims of the '698 Patent under 35 U.S.C. § 271(c).

175. IONpath commercialized its MIBIscope instrument that "that uses secondary ion mass spectrometry to image antibodies tagged with isotopically pure elemental metal reporters in intact tissue sections" combined with "time-of-flight mass spectrometry," the operation of which results in the MIBIscope users infringing upon the system covered under one or more claims of the '698 Patent including, but not limited to, the system as set forth in, for example, claim 1 of the '698 Patent.

176. On information and belief, beginning in 2018 or earlier, with an expected commercial launch date in 2019, IONpath knowingly, intentionally, and willfully began marketing and selling the infringing MIBIscope machine. The MIBIscope is a physical apparatus containing the elements of Fluidigm's patented system.

177. On information and belief, the MIBIscope is a "purpose-built" machine that does not serve any non-infringing use, but instead corresponds to the system of at least Claim 1 of the '698 patent.

178. IONpath has commercialized MIBItags which are isotopically labelled antibodies that can be used to provide the sample for analysis with the system of at least Claim 1 of the '698

patent. When analyzing tissue cells with the MIBIscope, including but not limited to in combination with the MIBItags, the user infringes upon a patented system as set forth in, for example, claim 1 of the '698 Patent.

179. On information and belief, beginning at least in 2019, IONpath began marketing and selling the infringing MIBItags. The MIBItags are isotopically labelled antibodies designed for implementation with Fluidigm's patented system.

180. On information and belief, the MIBItags do not serve any non-infringing use.

181. As a direct and proximate result of IONpath's infringement of the '698 Patent, Fluidigm has suffered and continues to suffer damage. Fluidigm is entitled to recover from IONpath Fluidigm's lost profits, and no less than a reasonable royalty, in an amount to be determined at trial.

182. As a direct and proximate result of IONpath's acts of infringement, Fluidigm has been irreparably harmed and will continue to be harmed unless and until IONpath's infringing acts are enjoined and restrained by order of this Court.

PRAYER FOR RELIEF

Wherefore, Fluidigm respectfully requests that this Court enter judgment and provide relief as follows:

A. Enter a judgment that IONpath has directly infringed and is directly infringing one or more claims of the '386 Patent, the '104 Patent, and the '698 Patent;

B. Enter a judgment that IONpath has indirectly infringed and is indirectly infringing by inducing others to infringe on ore more claims of the ‘386 Patent, the ‘104 Patent, and the ‘698 Patent;

C. Enter a judgment that IONpath has contributed and is contributing to the infringement of one or more claims of the ‘386 Patent, the ‘104 Patent, and the ‘698 Patent;

D. That Fluidigm be awarded damages against IONpath pursuant to 35 U.S.C. § 284 that are adequate to compensate Fluidigm for IONpath’s infringement, or inducement thereof, of Fluidigm’s ‘386 Patent, ‘104 Patent, and ‘698 Patent, but in no event less than a reasonable royalty for the use made of the claimed inventions together with interest and costs;

E. Permanently enjoin, under 35 U.S.C. § 283, IONpath, its officers, agents, servants, employees, attorneys, successors, and assigns and all other persons in active concert or participation with any of them from infringing the ‘386 Patent, ‘104 Patent, and ‘698 Patent;

F. Permanently enjoin, under 35 U.S.C. § 283, IONpath, its officers, agents, servants, employees, attorneys, successors, and assigns and all other persons in active concert or participation with any of them from inducing third parties into infringing the ‘386 Patent, ‘104 Patent, and ‘698 Patent;

G. Declare that IONpath’s infringement was and is willful from the time it became aware of the infringing nature of its product and awarding treble damages for the period of such willful infringement of the ’386 Patent, ‘104 Patent, and ‘698 Patent, pursuant to 35 U.S.C. § 284;

H. Declare this an exceptional case within the meaning of 35 U.S.C. § 285 and award Fluidigm their reasonable attorneys' fees, costs, and expenses;

I. An accounting of all infringing sales and revenues, together with post judgment interest and prejudgment interest from the first date of infringement, of the '386 Patent, '104 Patent, and '698 Patent;

J. Award Fluidigm compensation, monetary damages and punitive damages for IONpath's intentional interference with Fluidigm's contractual relations, in an amount to be ascertained at trial, together with interest thereon;

K. Award Fluidigm pre-judgment and post-judgment interest on the damages awarded;

L. Award Fluidigm its costs and reasonable attorneys' fees, expenses and costs, incurred in connection with this action; and

M. Award Fluidigm any and all further legal and equitable relief that the Court may deem just and proper under the circumstances.

DEMAND FOR JURY TRIAL

Fluidigm hereby respectfully requests a trial by jury on all issues raised in this Complaint so triable by right pursuant to Rule 38(b) of the Federal Rules of Civil Procedure.

Dated: October 11, 2019

K. Lee Marshall
BRYAN CAVE LEIGHTON PAISNER LLP

By: /s/ K. Lee Marshall
K. Lee Marshall

Attorneys for Fluidigm Corporation and Fluidigm Canada Inc.

EXHIBIT A

US010180386B2

(12) **United States Patent**
Bandura et al.

(10) **Patent No.: US 10,180,386 B2**
(45) **Date of Patent: Jan. 15, 2019**

(54) **MASS SPECTROMETRY BASED MULTI-PARAMETRIC PARTICLE ANALYZER**

(71) Applicant: **Fluidigm Corporation**, South San Francisco, CA (US)

(72) Inventors: **Dmitry R. Bandura**, Ontario (CA); **Vladimir I. Baranov**, Ontario (CA); **Scott D. Tanner**, Ontario (CA)

(73) Assignee: **Fluidigm Corporation**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **15/888,871**

(22) Filed: **Feb. 5, 2018**

(65) **Prior Publication Data**

US 2018/0195948 A1 Jul. 12, 2018

Related U.S. Application Data

(60) Continuation of application No. 13/294,799, filed on Nov. 11, 2011, now Pat. No. 9,952,134, which is a (Continued)

(51) **Int. Cl.**
H01J 49/04 (2006.01)
G01N 15/14 (2006.01)
(Continued)

(52) **U.S. Cl.**
CPC ***G01N 15/1404*** (2013.01); ***H01J 49/004*** (2013.01); ***H01J 49/04*** (2013.01);
(Continued)

(58) **Field of Classification Search**
None
See application file for complete search history.

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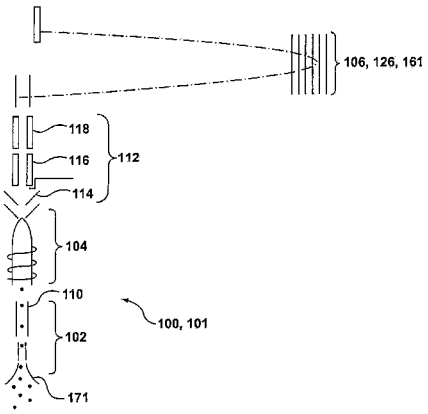
Primary Examiner — Andrew Smyth
(74) *Attorney, Agent, or Firm* — Kilpatrick Townsend and Stockton LLP

(57) **ABSTRACT**

A method for cellular analysis of cellular particles tagged with elemental tags, such as lanthanide-based elemental tags. Particles or element tags associated with particles can be vaporized, atomized, and ionized, such as with an inductively coupled plasma device or a glow discharge device. The vaporized, atomized, and ionized particles or element tags can be analyzed using mass spectrometry, such as using a time of flight mass spectrometer or a magnetic sector mass spectrometer. The amount of at least one element in individual particles can be measured through mass analysis. The amount of many different tags, for example at least five different tags, can be measured at the same time to facilitate multi-parametric analysis of cells and other particles. The vaporized, atomized, and ionized particles or element tags can be pretreated in an ion pretreatment system to filter out low mass ions, such as using a high-pass mass filter or a bandpass mass filter, to allow the elemental tags to pass therethrough.

20 Claims, 11 Drawing Sheets



Related U.S. Application Data

continuation of application No. 12/332,812, filed on Dec. 11, 2008, now abandoned, which is a division of application No. 11/089,023, filed on Mar. 25, 2005, now Pat. No. 7,479,630.

(60) Provisional application No. 60/555,952, filed on Mar. 25, 2004.

(51) **Int. Cl.**
H01J 49/00 (2006.01)
G01N 15/00 (2006.01)

(52) **U.S. Cl.**
CPC .. ***H01J 49/0431*** (2013.01); *G01N 2015/0065* (2013.01); *G01N 2458/15* (2013.01)

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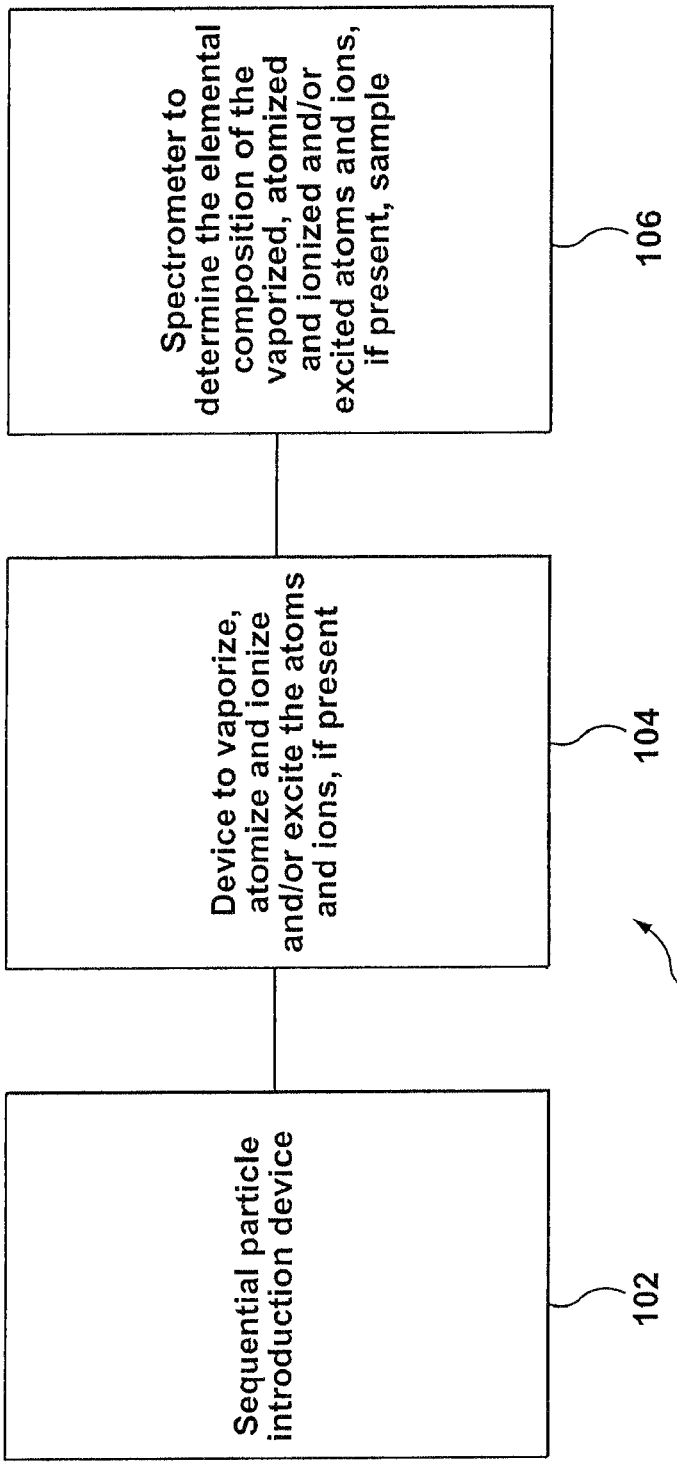
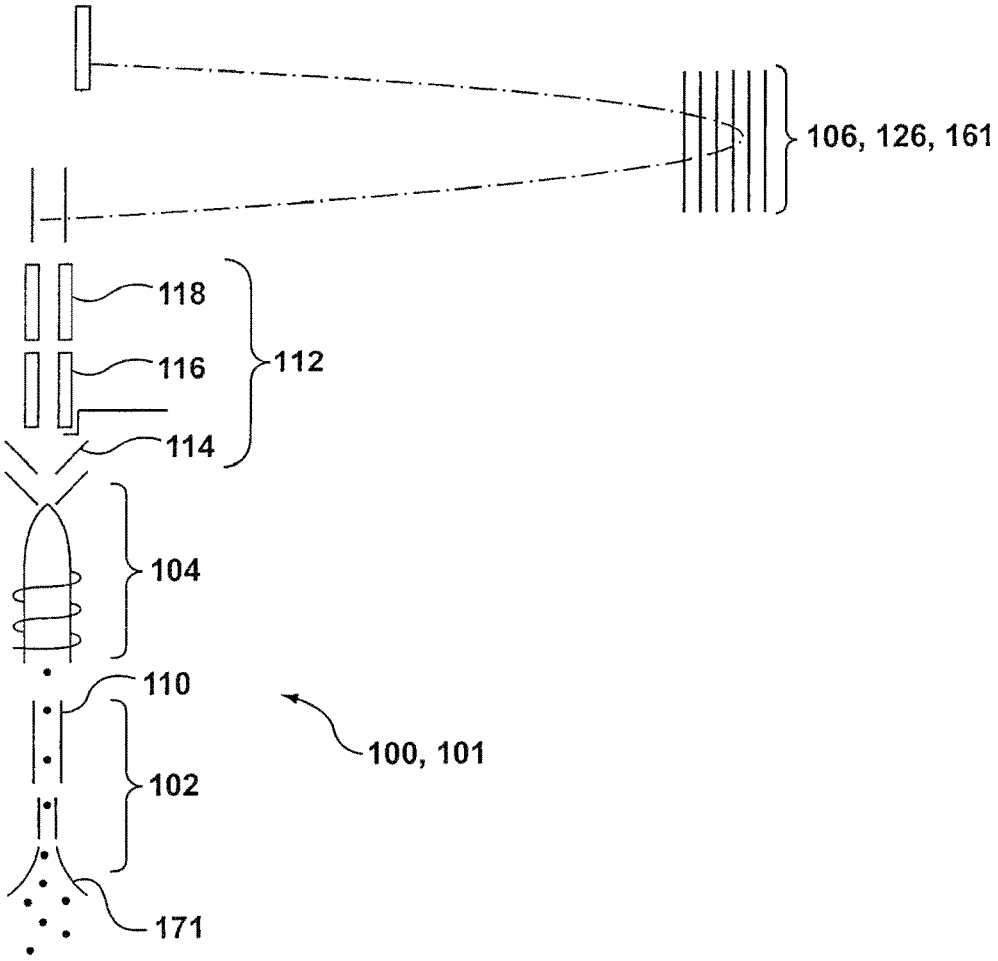

Spectrometer to determine the elemental composition of the vaporized, atomized and ionized and/or excited atoms and ions, if present, sample
106
Device to vaporize, atomize and ionize and/or excite the atoms and ions, if present
104
100
Sequential particle introduction device
102

FIG. 1



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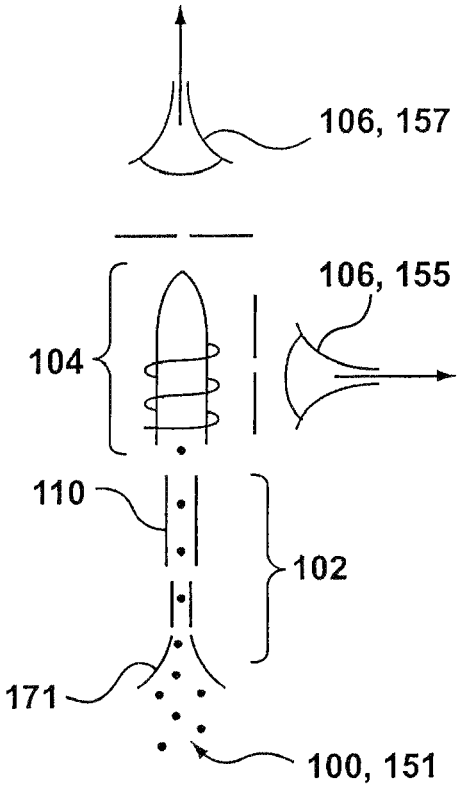

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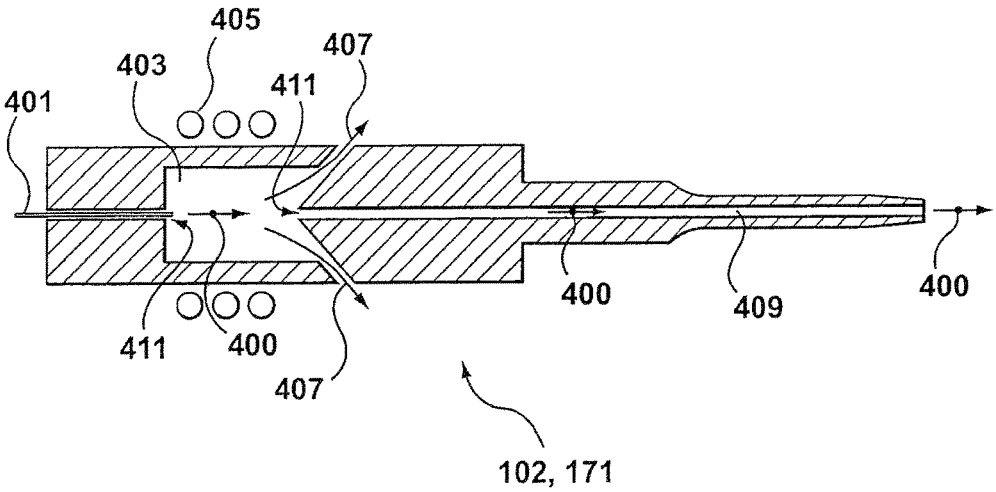
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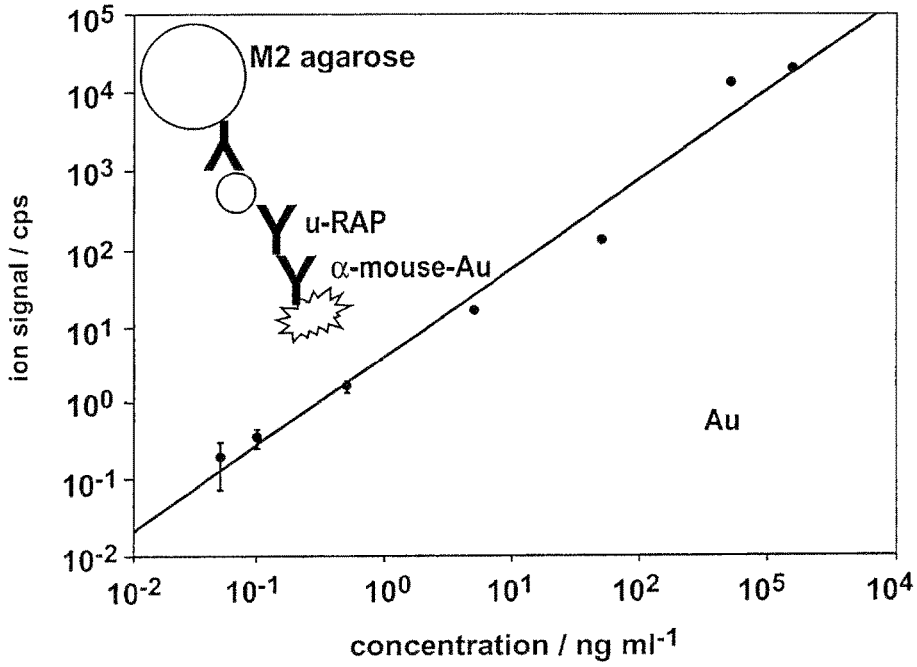
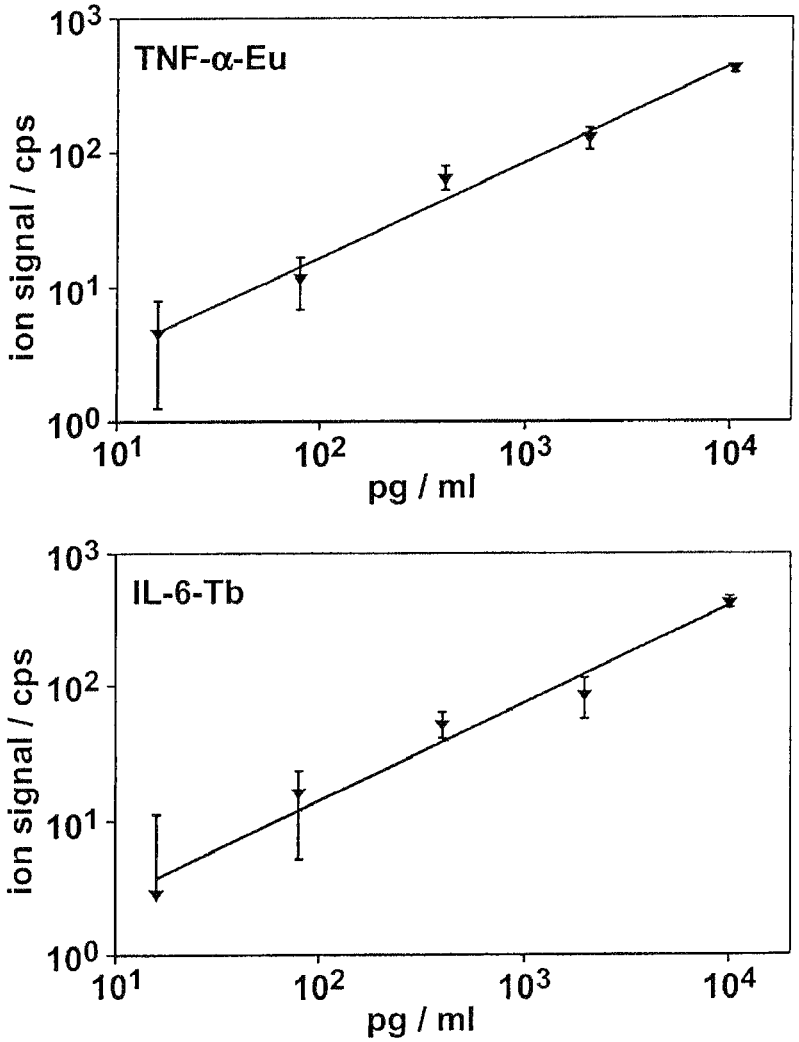

M2 agarose
u-RAP
α-mouse-Au
Au
ion signal / cps
concentration / ng ml-1
10-2
10-1
100
101
102
105
104
103

FIG. 5



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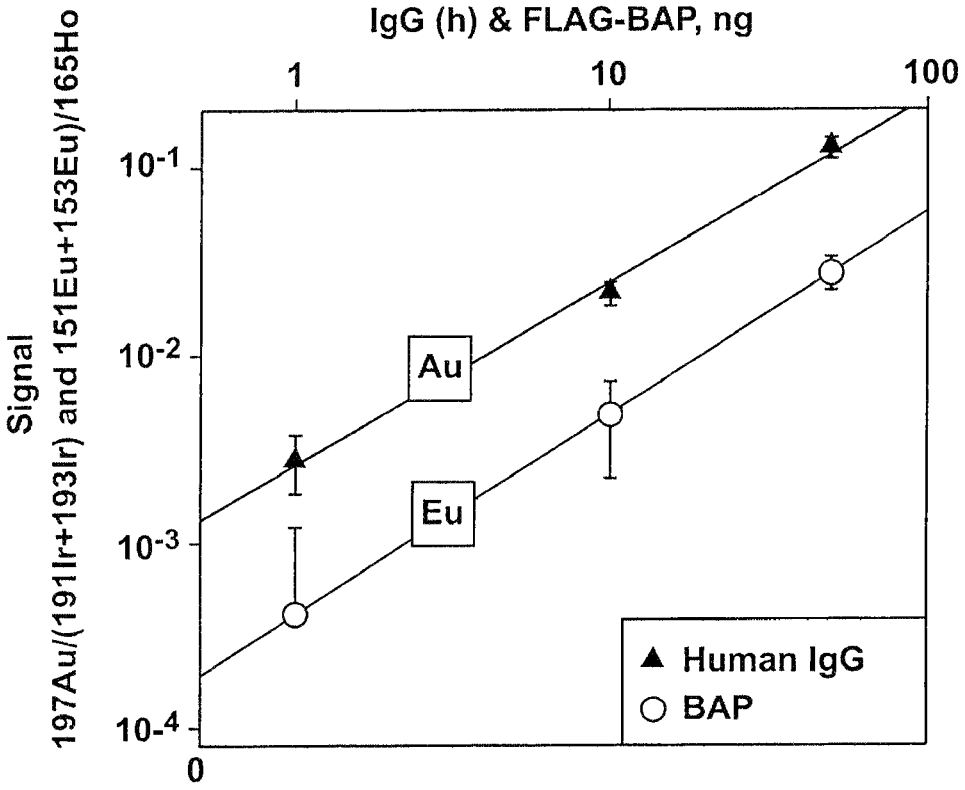
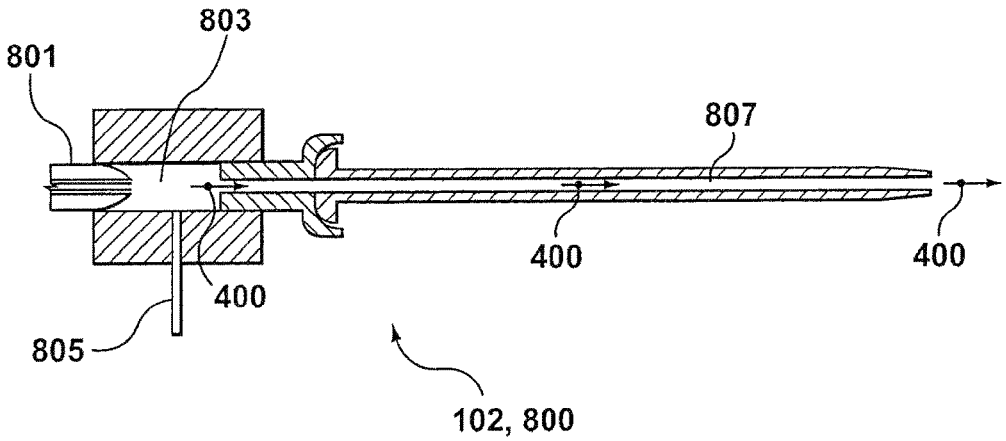

IgG (h) & FLAG-BAP, ng
1
10
100
Signal
197Au/(191Ir+193Ir) and 151Eu+153Eu)/165Ho
10-1
10-2
10-3
10-4
0
Au
Eu
Human IgG
BAP

FIG. 7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807
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805
102, 800

FIG. 8

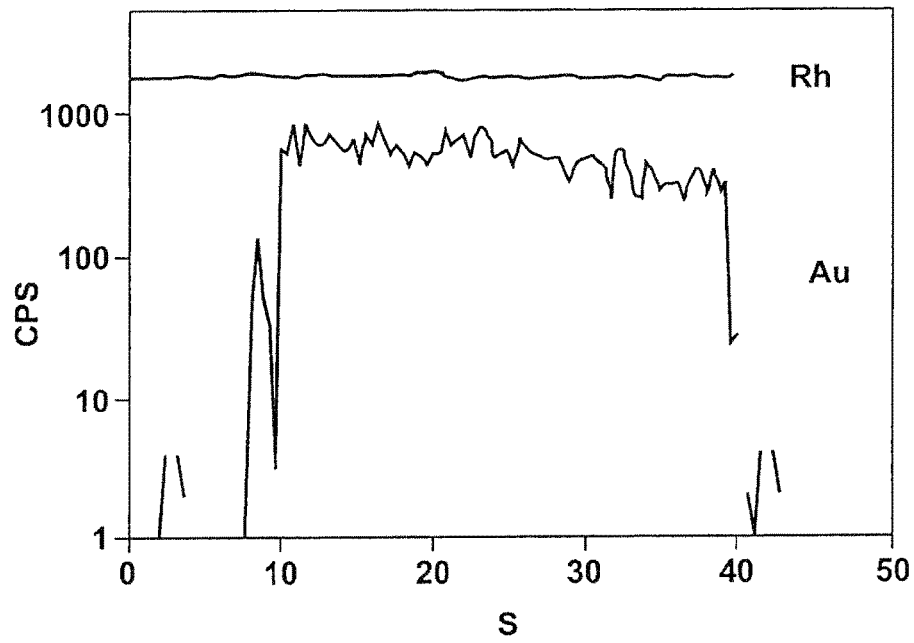
Rh
Au
1000
100
10
1
CPS
0
10
20
30
40
50
S

FIG. 9

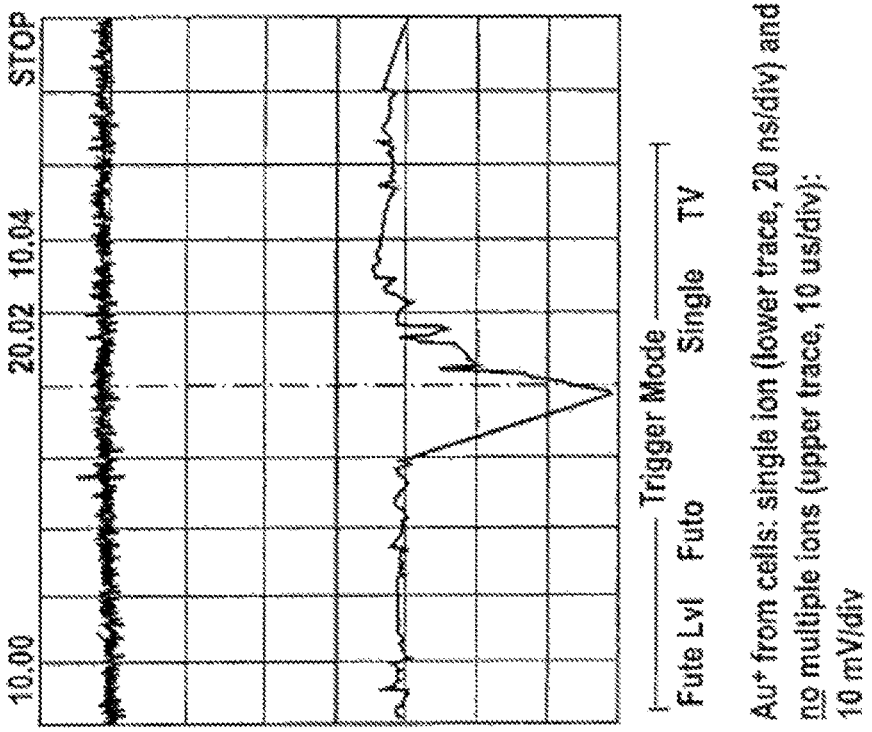

FIG. 10B

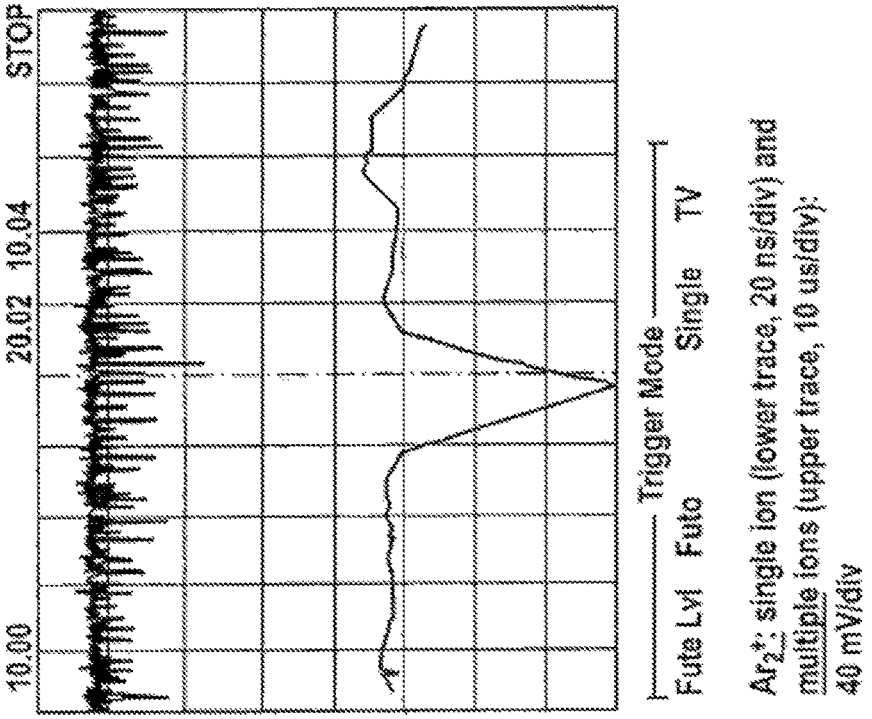

FIG. 10A

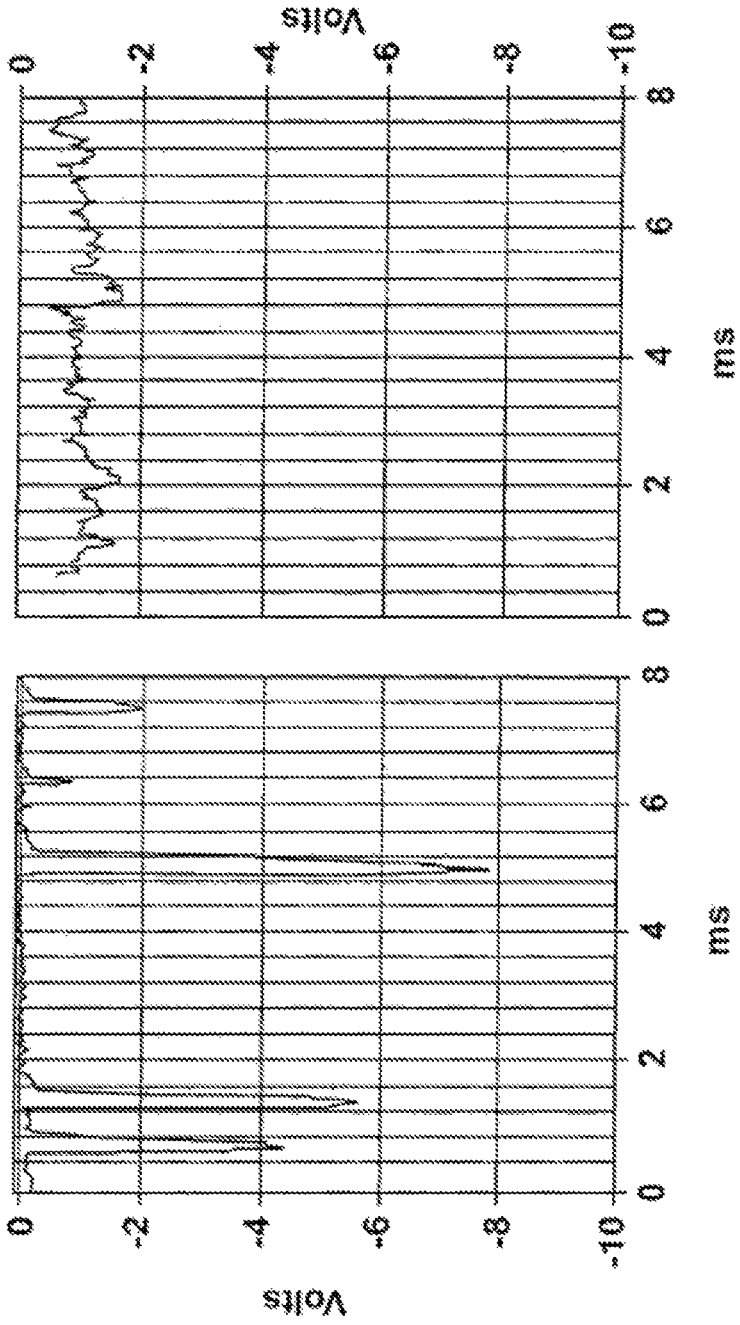

FIG. 11B

FIG. 11A

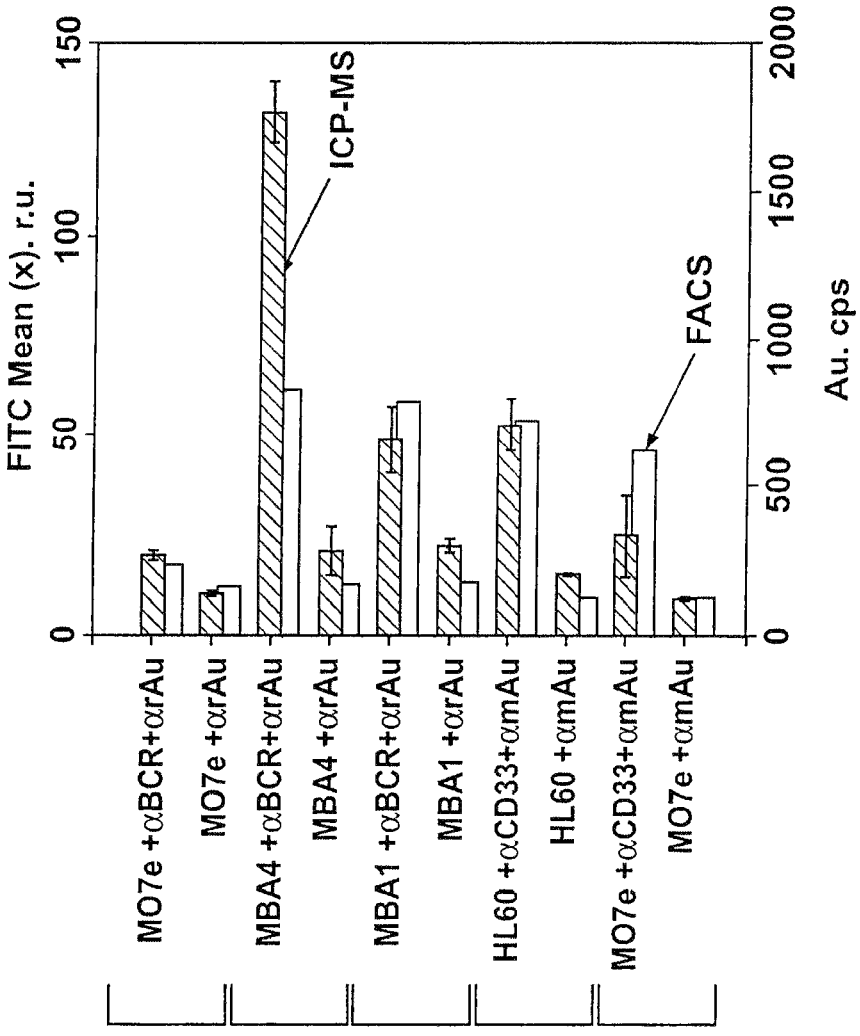
FITC Mean (x). r.u.
0
50
100
150
Au. cps
0
500
1000
1500
2000
ICP-MS
FACS
MO7e +αBCR+αrAu
MO7e +αrAu
MBA4 +αBCR+αrAu
MBA4 +αrAu
MBA1 +αBCR+αrAu
MBA1 +αrAu
HL60 +αCD33+αmAu
HL60 +αmAu
MO7e +αCD33+αmAu
MO7e +αmAu

FIG. 12

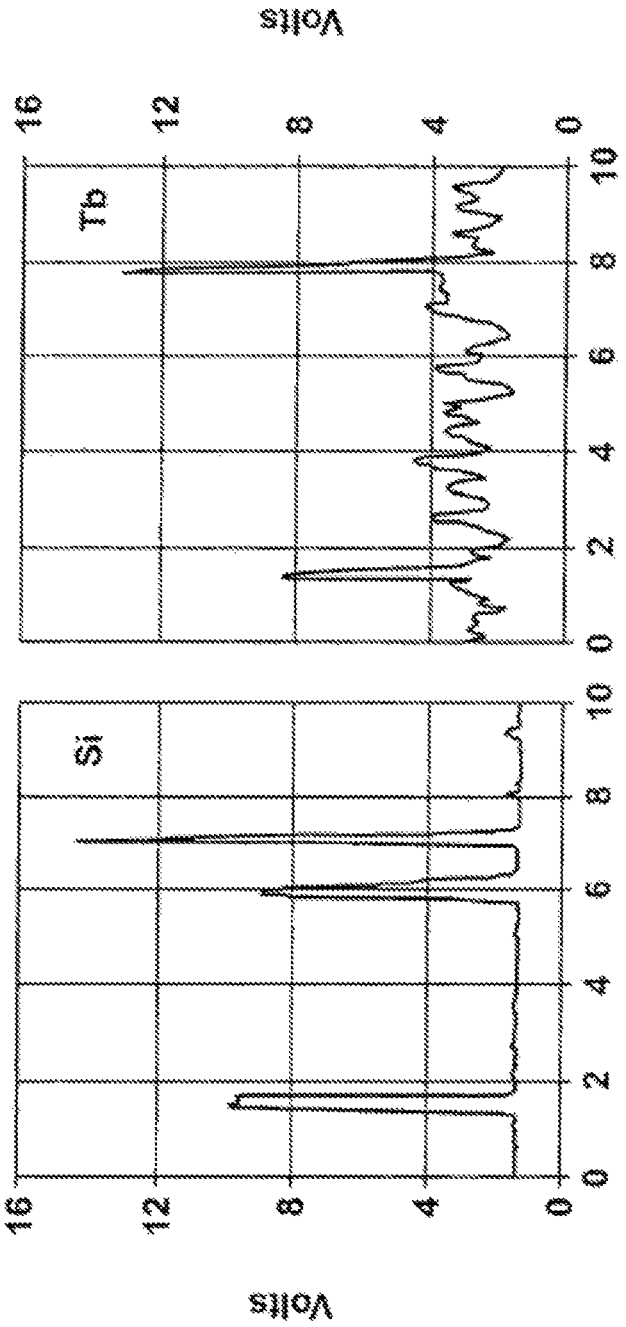

FIG. 13B

FIG. 13A

MASS SPECTROMETRY BASED MULTI-PARAMETRIC PARTICLE ANALYZER

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 13/294,799, filed Nov. 11, 2011, which is a continuation of U.S. patent application Ser. No. 12/322,812, filed Dec. 11, 2008, which is a divisional of U.S. Pat. No. 7,479,630, issued Jan. 20, 2009, which is a non-provisional of U.S. Patent Application Ser. No. 60/555,952, filed Mar. 25, 2004, all of which are hereby incorporated by reference.

The entire contents of U.S. patent application Ser. No. 09/905,907, filed Jul. 17, 2001 and entitled Elemental Analysis of Tagged Biologically Active Materials (published as US 2002/0086441); and Ser. No. 10/614,115, filed Jul. 3, 2003 and entitled Elemental Analysis of Tagged Biologically Active Materials (published as US 2004/0072250) are hereby incorporated by reference.

The entire contents of U.S. Pat. No. 6,524,793, filed Jun. 18, 1999 and entitled Multiplexed Analysis of Clinical Specimens Apparatus and Method; International Patent Application Publication WO 98/33203, published Jul. 30, 1998, and entitled Gate for Eliminating Charged Particles in Time of Flight Spectrometers; and each of the publications cited in the Reference Section herein are hereby incorporated by reference.

FIELD OF THE INVENTION

The invention features apparatus and methods for sequentially analyzing particles, for example single cells or single beads, by spectrometry. In particular, the invention provides elemental-flow cytometers.

BACKGROUND OF THE INVENTION

The ability to analyze single particles, for example single cells or single beads, is a useful tool in the health sciences, in human and animal food sciences, in environmental sciences, forensic sciences, and in genomics and proteomics.

In the health sciences, cells are recognized as members of certain classes, for example normal cells or cancerous cells for diagnostic or biomedical research purposes. Cells carry multiple antigens or biomarkers [1], either extracellularly or intracellularly [2], which can be quantified or qualified for clinical medicine [3] or biomedical research [4] purposes. These methods are useful for development of pharmaceutical products particularly in the development of cell based assays and toxicity studies.

For example, chronic lymphocytic leukemia (CCL) is recognized as a unique disorder of B-cells [5, 6]. CCL is a disease with an uncertain clinical picture, and is often misdiagnosed resulting in inadequate treatment. However, a more detailed study of a patient's cellular immunophenotypic profile allows reclassification of the patient, which leads to a more personalized diagnosis and treatment. Such classification requires multi-targeted analysis of many markers on a cell membrane as well as in-cell antigens, their qualitative and quantitative description, and consideration of minute concentration variances.

Other examples in the health sciences include the analysis of single cells in the subclassification of non-Hodgkin's lymphoma. In addition, single cell analysis is useful in immunophenotyping of helper T-cells, and the determination of the ratio of CD4 to CD8 T-cells, for indication of the HIV progression in HIV positive patients. Further, the technique can be used to analyze single cells from patients with renal, cardiac and bone marrow transplants, for discriminating between graft rejections and viral infections in post-operative patients.

In human and animal food sciences, the analysis of single cells can be used to detect artificial hormones, pesticides, herbicides or antibiotics. Finally, in environmental sciences, the analysis of single cells can detect toxic waste, for example, in plant or bacterial cells.

A known method of analyzing single cells is by a fluorescence activated cell sorter (FACS). FACS is a technology to measure biological properties of cells by scanning single cells as they pass through a laser beam. Cells are usually stained with one or more fluorescent dyes specific to cell components of interest, for example, receptors on the cell surface and DNA of the cell nucleus, and the fluorescence of each cell is measured as it traverses the excitation beam. Since the amount of fluorescence emitted is proportional to the amount of fluorescent probe bound to the cell antigen, antibodies conjugated to fluorochromes are routinely used as reagents to measure the antigen both qualitatively and quantitatively on and in the cell. Primarily, researchers use the sorting function of the FACS machines to investigate cell receptors and other membrane antigens on a specific cell population. It can be used for antibody screening in multiple cell lines simultaneously (for example, a transfected cell line expressing the antigen of interest and a control cell line not expressing the antigen). In its simplified flow cytometry function, FACS machines are used mostly without sorting, which allows for example the use of fixed permeabilized cells and analysis of intracellular antigens. Many routine flow cytometry methods that identify antigens expressed on the cell surface and within the cell using specific antibodies, as well as general immunoassay methods for clinical diagnostics and treatment have been developed. Some of them involve multiplexing through the use of different fluorochromes and lasers. Deficiencies of this approach are related to limitations and difficulties of cell staining methods and spectral overlap of fluorochromes. Other measurable optical parameters include light absorption and light scattering, the latter being applicable to the measurement of cell size, shape, density, granularity, and stain uptake.

U.S. patent application Ser. No. 09/905,907, published under US 2002/0086441 on Jul. 4, 2002, and Ser. No. 10/614,115, describe labeling of analytes for analysis by mass spectrometry. Biologically active materials (for example, antibodies and aptamers) are labeled and conjugated to analytes prior to analysis.

SUMMARY OF THE INVENTION

In one broad aspect, the present invention provides an apparatus for introducing particles sequentially and analyzing the particles (for example, single particles such as single cells or single beads), by spectrometry. The apparatus, an elemental flow cytometer, is an instrument comprising: a means for introducing single particles sequentially, a means to vaporize, atomize, and excite or ionize the particles or an elemental tag associated with an analyte on the particles, and a means to analyze the elemental composition of the vaporized, atomized, ionized and/or excited particles, or an elemental tag associated with the particles.

It is to be understood that although the term "means for introducing single particles sequentially" is used, this may

encompass introduction of a predetermined number of particles (for example, 2 or more) in discrete 'packets'.

It is also to be understood that the term "means to vaporize, atomize, and excite or ionize" includes means where atomization may not be necessary, so that the term may or may not encompass vaporization followed by ionization directly. In some applications, such as for example optical emission spectrometry (OES), it is not essential to ionize the sample; emission from atomic species can be sufficient. For OES, it is necessary only to excite the atoms (or ions) to cause emission. Thus, for example, "vaporize, atomize and ionize" should be understood to mean vaporize, atomize and ionize (for mass spectrometry) or excite (either or both atoms and ions) for OES.

Another aspect of the invention is an analytical instrument. The instrument has a sample introduction system for generating a stream of particles from a sample. An ionization system receives particles in the stream. The ionization system is operable to atomize particles in the stream as the particles are received from the sample introduction system and to ionize atoms from the atomized particles. The instrument has an ion pretreatment system and a mass analyzer. The ion pretreatment system is adapted to transport ions generated by the ionization system to the mass analyzer. The mass analyzer is adapted measure the amount of at least one element in individual particles from the stream by performing mass analysis on the ions from the atomized particles.

Another aspect of the invention is an instrument for performing multi-parametric quantitative analysis of particles in a stream of particles. The instrument has a sample introduction system for generating a stream of particles from a sample. A particle analyzer is adapted to measure the amount of each of a plurality of at least five different tags in each of a plurality of particles in the stream of particles produced by the sample introduction system. The particle analyzer has a detector adapted to generate signals corresponding to each tag. The signals generated by the detector corresponding to each of the tags is independent from the signal generated by the detector corresponding to the others of the tags.

In another broad aspect, the invention provides a method for analyzing particles that have been introduced sequentially, such as single cells or single beads, by spectrometry. A trigger will report the ion cloud arrival with following analysis, including for example initiation of data acquisition. Triggering may be based, for example on light scattering or on an ion current change or ion composition change.

Another aspect of the invention is an elemental flow cytometer, comprising: a means for introducing particles sequentially into a device to vaporize, atomize and excite or ionize the particles, or an elemental tag associated with the particles; a device to vaporize, atomize and excite or ionize the particles, or an elemental tag associated with the particles, downstream of the means for introducing particles sequentially; and a spectrometer to analyze the vaporized, atomized and ionized and/or excited particles, or the elemental tag associated with the particles.

Another aspect of the invention is a mass-spectrometer-based flow cytometer, comprising: a means for introducing particles sequentially into a device to vaporize, atomize and ionize the particles, or an elemental tag associated with the particles; a device to vaporize, atomize and ionize the particles, or an elemental tag associated with the particles, downstream of the means for introducing particles, sequentially; and a mass spectrometer operatively connected and downstream of the device to vaporize, atomize and ionize.

Another aspect of the invention is a mass-spectrometer-based flow cytometer, comprising: a means for introducing particles sequentially into a device to vaporize, atomize and ionize the particles, or an elemental tag associated with the particles; a device to vaporize, atomize and ionize the particles, or an elemental tag associated with the particles, downstream of the means for introducing particles sequentially; an ion pretreatment device operatively connected and downstream of the device to vaporize, atomize and ionize; and a mass spectrometer operatively connected and downstream of the ion pretreatment device. The ion pretreatment device may be provided as a part of the mass spectrometer, preferably upstream of the mass anaylzer section thereof.

Another aspect of the invention, is an optical emission spectrometer-based flow cytometer, comprising: a means for introducing particles sequentially into a device to vaporize, atomize and excite or ionize the particles, or an elemental tag associated with the particles; a device to vaporize, atomize and excite or ionize the particles, or an elemental tag associated with the particles downstream of the means for introducing particles sequentially, and an optical emission spectrometer to analyze the vaporized, atomized and excited or ionized particles, or the elemental tag associated with the particles downstream of the device to vaporize, atomize and excite or ionize the particles.

Another aspect of the invention, is a method of analyzing particles that have been introduced sequentially into a device to vaporize, atomize and excite or ionize, comprising: sequentially introducing particles or particles associated with an elemental tag, into a device to vaporize, atomize and excite or ionize the particles or the elemental tag associated with the particles; and introducing the vaporized, atomized and excited or ionized particles, or the elemental tag associated with the particles into a spectrometer.

The labeling or tagging of the single particles with elemental tags can be done, for example, using the methods and system disclosed in U.S. Ser. No. 09/905,907 and U.S. Ser. No. 10/614,115, both applications of which are herein incorporated by reference. U.S. Ser. No. 09/905,907 and U.S. Ser. No. 10/614,115 describe methods and systems for the analysis of biologically active materials conjugated to analytes by mass spectrometry. Other methods of labeling or tagging the particles will also serve. If, for example, the particles are beads, the particles themselves can be labeled either on the surface or within their bodies, as disclosed herein.

Another aspect of the present invention is to provide kits having reagents for carrying out the methods of the present invention and instructions for these methods.

Another aspect of the present invention is to provide beads with an affinity substance as a carrier to measure an analyte in a sample, further comprising an elemental label or tag. The elemental tag can be on the analyte, on the affinity substance or (and) on or in the bead itself.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a schematic diagram of a flow cytometer according to the invention.

FIG. **2** is a schematic diagram of an embodiment of a mass-spectrometer-based flow cytometer according to the invention.

FIG. **3** is a schematic diagram of an embodiment of an optical emission spectrometer (OES)-based flow cytometer of the invention.

FIG. **4** is a schematic diagram of a single-particle injector according to the invention.

FIG. **5** is a calibration curve for flag-BAP using agarose bead immobilization with α-BAP primary and Au-tagged α-mouse secondary antibodies.

FIG. **6** is a plot of Fluorokine bead assay, detecting TNF-.alpha. and IL-6 simultaneously using distinguishable (Eu and Tb) elemental tags on the corresponding primary antibodies.

FIG. **7** is a plot of an ELISA based assay coupled to ICP MS showing the simultaneous quantitation of two proteins.

FIG. **8** shows a schematic diagram of a sample introduction system

FIG. **9** shows overlaid results of measuring ion signals as a function of time for direct injection of a standard solution of 100 ppt Rh (1% HNO_3) and of a MOTE cell suspension for which the surface antigen CD33 was tagged with a Au particle.

FIG. **10**A shows oscilloscope output of an Ar_2^+ signal from MO7e cell introduction for which the surface antigen CD33 was tagged with a Au particle.

FIG. **10**B shows oscilloscope output of an Au^+ signal from MO7e cell introduction for which the surface antigen CD33 was tagged with a Au particle.

FIG. **11**A shows an analog signal from an oscilloscope registered while continuously monitoring Na^+, in a cell suspension in a 30 mM $CaCl_2$ buffer.

FIG. **11**B shows an analog signal from an oscilloscope registered while continuously monitoring Na^+, for a 30 mM $CaCl_2$ buffer.

FIG. **12** shows comparative data for analysis of cell surface proteins and intracellular proteins by both conventional FACS and by the method of the present invention.

FIG. **13**A shows Si^+ signal for stober silica particles grown in the presence of a Tb solution.

FIG. **13**B shows Tb^+ signal for stober silica particles grown in the presence of a Tb solution.

DEFINITIONS

ICP-MS: is an Inductively-Coupled Plasma Mass Spectrometer.

FACS: is a Fluorescence Activated Cell Sorter.

Various aspects of the present disclosure are described herein with reference to single particles. However, in some cases, these aspects of the present disclosure can be used with packets of a predetermined number of discrete entities (e.g., 2, 3, or 4). Various aspects of the present disclosure as described herein can be used with single cells, single beads, single bacteria, single viral particles, single pollen particles, single microscopic insects such as dust mites.

Tag (or label): a chemical moiety that provides a distinguishable signal of the presence of the analyte or analyte complex with which it is associated, as for example through linkage to an affinity product that in tum recognizes the analyte or analyte complex. As disclosed herein, the tag (which is also called an "elemental tag") can contain an element or an isotope (or multiple copies thereof) that provide the distinguishable signal. A tag can include for example an element or isotope of an element that is associated with an analyte or analyte complex and which is measured to determine the presence of the analyte. A tag can also include, for example, any distinguishable component (e.g., an element or isotope or multiple copies thereof) that is provided on the surface or within the body of, or is otherwise associated with, a particle and serves to distinguish that particle from other particles.

TOF-MS: is a Time-of-Flight Mass Spectrometer

DESCRIPTION OF SPECIFIC EMBODIMENTS INCLUDING THE BEST MODE CURRENTLY CONTEMPLATED BY THE INVENTORS

The elemental flow cytometer of the present invention provides for the identification and quantitative analysis of particles that have been introduced sequentially into a device to vaporize, atomize and excite or ionize them, for example individual cells or microscopic beads, by measuring the elemental composition of the single particle (or any distinctive part of cell or bead), or a tag or label associated with an analyte located on or in the cell or bead by employing the mass-to-charge ratio or optical emission of the disintegrated tag elements. The tag can be of any chemical nature, as it is only its elemental composition that is important. In comparison, the chemical structure of the appropriate tag is absolutely critical to provide a unique fluorescence in FACS.

The elemental flow cytometer includes:

- means for introducing particles sequentially (for example, cell-by-cell or bead-by-bead), preferably adapted for discrete event analysis;
- means to vaporize, atomize and excite or ionize the particles,
- or an elemental tag (or classifiable elemental composition) associated with an analyte of interest on or in the particles to quantify the analyte of interest associated with the particles;
- and means for registering the information on elemental composition of the particles, or an elemental tag associated with an analyte on the particles. This can be done, for example, by mass spectrometry (MS) or by optical emission spectrometry (OES).

Elemental flow cytometers according to the invention are quantitative analytical instruments [7]. They can perform the task of quantitative or qualitative analysis of biological or environmental samples using analytical methods [8].

Beads with an affinity substance can be used as carriers to measure an analyte in a sample. The placement of the elemental tag or label can be on the analyte, on the affinity substrate, and/or on or in the bead itself.

Specific embodiments of the elemental flow cytometer include: (1) a mass spectrometer based flow cytometer (MS FC) and (2) an optical emission spectrometer based flow cytometer (OES FC).

A mass spectrometer based flow cytometer (MS FC) comprises:

- means for introducing particles sequentially:
- means to vaporize, atomize and ionize the particles and/or any tags that may be associated with the particles; and
- a mass spectrometer to analyze the elemental composition of the vaporized, atomized and ionized particles, and/or any tags that may be associated with the particles.

MS FCs according to the invention can further comprise ion pretreatment devices, for pretreatment of ions prior to analysis by the mass spectrometer.

The means to vaporize, atomize and ionize the single particles may include glow discharge, graphite furnace, and capacitively coupled plasma devices, or other suitable devices. Preferably, the means to vaporize, atomize and ionize the single particle includes an inductively coupled plasma (ICP) device because it has a capacity to disintegrate, vaporize, atomize and ionize cells and beads during their short residence time in the plasma and because the ICP is particularly tolerant of concomitant materials, is robust to

changes of the composition of the plasma gases, and is a highly efficient atomizer and ionizer.

The ion pretreatment device acts, inter alia, as an interface between atmospheric conditions in the vaporizer/atomizer/ionizer and the vacuum in the mass spectrometer. In addition, the very strong ion current originating from this source is dominated by space charge, which could be reduced by an accelerating potential and/or by rejection of major plasma ions on the basis of their mass-to-charge ratio (Ar^+, for example). In the case of a TOF MS, the ion pretreatment device also conditions the ion flow for the needs of the TOF mass analyzer. For example, it will narrow the ion energy distribution and focus the parallel ion beam close to the axis of the mass analyzer.

The mass spectrometer can be any mass spectrometer. For example, it can be a quadrupole, magnetic sector with array detector, 3D Ion Trap or Linear Ion Trap mass spectrometer. Preferably it is a time of flight mass spectrometer (TOF MS). TOF MS is a simultaneous analyzer. It is able to register all masses of interest in one particle simultaneously.

The optical emission spectrometer based flow cytometer (OES FC) comprises:

- a means for introducing particles sequentially; a means to vaporize, atomize and excite or ionize the particles, and/or any tags that may be associated with the particles; and
- an optical emission spectrometer to analyze the elemental composition of the vaporized/atomized and excited or ionized particles and/or any tags that may be associated with the particles.

The means to vaporize, atomize and excite or ionize the single particles may include glow discharge, graphite furnace, and capacitively coupled plasma devices, or other suitable devices. Preferably, the means to atomize and ionize the single particles includes an inductively coupled plasma (ICP) device because it has a capacity to disintegrate, atomize and excite or ionize cells and beads during their short residence time in the plasma and because the ICP is particularly tolerant of concomitant materials, is robust to changes of the composition of the plasma gases, and is a highly efficient atomizer and ionizer.

Processes implemented by elemental flow cytometers according to the invention can also include an in-line lysis step between the means for single particle introduction and the means to vaporize, atomize and ionize.

The embodiments will now be described in detail.

In a most general aspect, the present invention provides an elemental analyzer as a detector for a flow cytometer. FIG. **1** shows schematically a cytometer **100** suitable for use implementing methods of analysis according to the invention. Cytometer **100** comprises means **102** for introducing particles sequentially, for example a cell or particle injector **171** (FIGS. **2**, **3**, **4**), operatively connected upstream of a device **104** for vaporizing, atomizing and exciting or ionizing particles or elemental tags associated with the particles. The elemental composition of the particle or elemental tag is determined by a spectrometer **106** operatively connected to the device **104**. Spectrometer **106** may, for example, include an optical spectrometer **157**, which detects the emission from excited atoms and/or ions, or a mass spectrometer **116** which detects the ions.

In one embodiment the present invention provides a mass-spectrometer based flow cytometer (MS FC) **101**. A schematic representation of such an embodiment is given in FIG. **2**.

Referring to FIG. **2**, mass-spectrometer based embodiment **101** of cytometer **100** comprises means **102** for introducing particles sequentially, for example a cell or particle injector **171**, operatively connected upstream of device **104** for vaporizing, atomizing and exciting or ionizing particles or elemental tags associated with the particles, namely an inductively coupled plasma (ICP) vaporizer/atomizer/ionizer. In the embodiment shown, means **102** comprises optional in-line lysis system **110**.

Ion pretreatment device **112**, in this instance comprising vacuum interface **114**, high-pass filter **116** and gas-filled "cooler" cell **118**, is operatively connected downstream of the ICP vaporizer/ionizer.

Time-of-flight (TOF) mass spectrometer **106**, **161**, **126** is operatively connected downstream of the ion pretreatment device. Use of mass spectrometer-based cytometer **101** according to such embodiments to analyze single particles can provide greatly improved accuracy, large dynamic range and high sensitivity, compared to prior art systems. In addition, because a large number of distinguishable elements and isotopes can be used as tags, and because the mass spectrometer provides high abundance sensitivity (exceedingly small overlap of signal on adjacent mass/charge detection channels), it facilitates a higher order of multiplexing (simultaneous determination of multiple analytes, each distinguishably tagged) than prior art fluorescence-based detection flow cytometers. Further, because of the high resolution of adjacent mass/charge detection channels and the large linear dynamic range of the mass spectrometer, the instrument provides for a large dynamic range both for a given analyte and between analytes. Thus, in many instances generic tagging moieties can be used in analyses in which the copy-count of the analytes differs dramatically; this distinguishes the method from conventional fluorescence detection methods for which the composition of the several fluorophores used for multiplex assay must often be adjusted for a particular assay to provide emission intensities of similar magnitude to minimize spectral overlap. Thus such embodiments can provide researchers and clinicians substantially improved analytical and prognostic capabilities.

Another important application of cytometers according to this embodiment of the invention is to multiplex assay distinguishable beads, where the beads are distinguished by their elemental compositions and have attached affinity products that recognize an antigen in the sample into which they are introduced, where the antigen is then further recognized using a sandwich (or other) assay employing yet a further distinguishable element.

Significant components of the mass spectrometer-based flow cytometer **101** of FIG. **2** and methods of use will now be described in detail.

Tagging

In Certain Cases the Particle (for Example a Single Cell) Will Not Require Tagging

In some cases a particles will not require tagging. For example, if a single cell contains or is bound to an element that is detectable against the background by mass spectrometry, no tagging is required. For example, for the analyses of bacterial or plant cells that accumulate elemental species in bioremediation, additional tagging would not be required. Further, the intracellular accumulation of metal, for example platinum- or gold-containing drugs would not require additional tagging.

In Cases where Single Particles Require Tagging

Tagging of particles can be done by many methods as is known to those of skill in the art. For example, fluorescent dyes which have a succinimidyl ester moiety react efficiently with primary amines of proteins (antibodies) to form stable dye-protein conjugates. In a first step to tag DNA, an

amine-modified nucleotide, 5-(3-aminoallyl)-dUTP, can be incorporated into DNA using conventional enzymatic tagging methods. In a second step, the amine-modified DNA can be chemically tagged using an amine-reactive fluorescent dye. Biotinilation of antibodies can be carried out using sulfhydryl-directed solid-phase chemistry. These methods are well established and are available in kit formats from different companies, including for example Molecular Probes Inc, Pierce Chemical Company, and others. Specific chemical reactions are known in radioimmunochemistry. For example, radionuclides (88/90)Y and (177)Lu can be used to tag antibodies using cyclic diethylenetriaminepentaacetic acid anhydride (cDTPA), isothiocyanatob enzyl-DTPA (SCN-Bz-DTPA), or 1,4,7,10-tetraazacyclododecanetetraacetic acid (DOTA)(PMID: 14960657).

Elemental analysis of tagged biologically active materials has been disclosed in the incorporated references, U.S. patent application Ser. Nos. 09/905,907 and 10/614,115. Tagged biologically active materials, for example, antibodies and aptamers, etc., that react specifically with cellular components can be used to tag cells. Other affinity products are known to those skilled in the art. For example, they may include antigens, RNA, DNA, lipoproteins, glycoproteins, peptides, polypeptides, hormones, etc.

Although in many applications of systems and methods according to the invention it is convenient to tag each biologically active material (for example an antibody, aptamer or antigen) with a single element or isotope, it should be readily appreciated by those skilled in the art that an antibody or antigen may be tagged with more than one element. As there are more than 80 naturally-occurring elements having more than 250 stable isotopes, there are numerous elements, isotopes, and combinations thereof to choose from. Within limits prescribed by the need to have distinguishable tags when in combination, this will allow for simultaneous detection of numerous biologically-tagged complexes. It is advantageous if the relative abundance of the tag elements is sufficiently different from the relative abundance of elements in a given sample under analysis. By "sufficiently different" it is meant that under the methods of the present invention it is possible to detect the target antibody or antigen over the background elements contained in a sample under analysis. Indeed, the difference in inter-elemental ratios of the tagged antibody or antigen, and the sample matrix can be used advantageously to analyze the sample.

It is feasible to select elemental tags, which do not produce interfering signals during analysis. Therefore, two or more analytical determinations can be performed simultaneously in one sample. Moreover, because the elemental tag can be made containing many atoms, the measured signal can be greatly amplified.

The use of multiple copies of the element or isotope per tag can improve the sensitivity linearly, particularly, for example in the employment of ICP-MS embodiments of the invention. For multiplex assay of up to 23 simultaneous analytes, the tags can be conveniently constructed using the natural isotopic distributions of, for example, Ru, Rh, Pd, Ag, In, La, Ce, Pr, Nd, Sm, Eu, Th, Dy, Ho, Er, Tm, Yb, Lu, Hf, Re, Ir, Pt and Au. These elements, which are expected in most instances to be uncommon in biological samples, each have at least one isotope with natural abundance greater than 10% that is not significantly interfered by the others or by the oxide or hydroxide ions of the others. For those isotopes of lower natural abundance (e.g., ^{143}Nd, 12.2%), tagging with the isotopically enriched isotope provides an obvious sensitivity advantage. Where a higher order of multiplexing is desired, the use of commercially-available enriched isotopes (of which there may be as many as 167 of 55 elements that are not expected to be common in biological systems) offers a possibility (depending, of course, on availability, cost and isotopic purity). For example, there are as mentioned at least 35 isotopes of the lanthanides and noble metals alone that may be obtained in enriched form, are note expected to be common in biological systems and are largely independent with respect to mutual interference (though some care in the selection of the tagging protocol is to be taken where large differences in copy counts of the analytes occur; for example, if the copy count of an analyte tagged with .^{169}Tm is 1000 times greater than for an analyte tagged with .^{185}Er, .^{169}TmO$^+$ will interfere significantly with the determination of .^{185}Er$^+$ since TmO$^+$ is typically about 0.07% of the Tm$^+$ signal (though, as for FACS, some of this interference can be corrected mathematically since the fractional formation of oxide ions is stable and can be calibrated). In special circumstances, it might be feasible to tag a given biologically active material with more than one element or isotope (for example, there are in theory 20 distinguishable 3-isotope tags that can be constructed from 4 isotopes).

The invention allows the development of a novel powerful technique to measure biological properties of cells by analyzing single cells as they pass through an ICP. When using antibodies as the affinity product (biologically active material) the amount of a tag element detected by the mass spectrometer is proportional to the amount of tagged affinity product bound to the cell. Antibodies conjugated to the elemental tag are routinely used as reagents to measure the antigen both qualitatively and quantitatively, for example acquiring the patient's immunophenotypic profile, which is almost unlimited in the number of markers of interest. Another advantage offered by the invention is a reduced need to enhance the antibody signal by "sandwich" immunostaining" which can result in analytical errors.

Methods according to the invention are distinct from the approach of conventional methods (such as fluorescence, radioimmunoassay, chemiluminescent assay) that are challenged by overlap of detector signals, limited dynamic range, time-sensitive signals, and in some instances sensitivity. Accordingly, the method offers the potential for massively multiplexed assay (limited principally by the independence and cross-reactivity of the affinity chemistry) with essentially no signal overlap. Where the elemental (isotopic) tags are quantitatively associated with specific affinity products, the quantitative characteristic of ICP-MS offers a novel opportunity for absolute determination of multiple antigens simultaneously.

The method and apparatus can, for example, detect as few as 100 copies of each tag per cell. It is estimated that for the detection of as few as 100 copies of each tag per cell, at least 70 atoms per tag will be required.

The invention provides the feasibility to perform massively multiplexed bead assays. Current fluorescence-based flow cytometers are frequently used for bead assays. In this application, beads are typically labeled with 2 fluorochromes in varying ratios, typically providing up to about 100 distinguishable beads as determined by the fluorophore emission ratio (see, for example, the incorporated reference, U.S. Pat. No. 6,524,793 and references therein). Each bead also has attached affinity products (e.g., antibodies) that recognize an analyte in a solution in which the bead is placed, each bead of different "colour" having an affinity product for a different analyte. Once exposed to the sample solution, the captured analyte is then sandwiched with

another antibody having a third fluorophore reporter. Thus, in the flow cytometric analysis, the beads can be mixed, the copy-count of the analyte captured determined by the emission of the third fluorophore and the identity of the analyte determined by the ratio of the emissions of the bead-labeling fluorophores. Accordingly, the conventional fluorescence detector flow cytometer can perform multiplexed bead assays to as high 100 order (the number of distinguishable "colours", though in practice much fewer are used (presumably because of signal overlap, which limits the measurement accuracy (and thus confidence in the identification of the bead) when the ratio of the fluorescence emission intensity is large (e.g., one or two orders of magnitude, depending on the emission wavelength distributions).

A similar method can be implemented using mass spectrometer-based flow cytometers according to the invention, with the advantage that the degree of multiplexing can be vastly increased and the overlap of signals can be virtually eliminated from concern. For example, the bead can incorporate (either on its surface or, probably more conveniently, within its body, mixtures of elements or isotopes that can be used to report the identity of the bead. Assuming that the detector has a dynamic range of 3 orders of magnitude and that factors of 3 in relative signal can be reliably determined, 2 elements incorporated into the bead allows 63 distinguishable beads. Under the same assumptions but using 5 element labels provides 32,767 distinguishable beads, and if the dynamic range is 5 orders of magnitude, the same 5-element labels provide for 248,831 distinguishable beads. Furthermore, these few labeling elements can be selected so that signal overlap is nonexistent (e.g., by choosing them such that they appear at mass differences greater than a few atomic mass units), which enables the large dynamic range of detection. The sandwich assay for the analyte captured by the bead employs a yet different element tag, which also is readily distinguished from the bead-labeling elements. Further, in this configuration each bead can contain several affinity products to attach several different analytes per bead, each recognized by a sandwich assay using a yet different element, providing for multiplex assay both between beads and on a single bead. One anticipated application is for a 96-well plate (or 384-well, or 1536-well) for which a differently-labeled bead is provided to each well, and multiplexed element-tagged immunoassay on the bead surface in each well is conducted. The entire contents of the plate (96, or 384 or 1536 wells) can then be pooled and the result analyzed by flow cytometry, thus providing a type of mass spectrometer "plate reader" (where the bead identity, as determined by its elemental composition, identifies the well in which the assay took place).

Means for Introducing Particles Sequentially

The sample introduction system **102** can comprise several devices that are currently in use with other flow cytometry sample introduction systems. For example, there currently exist several cell or particle injector **171** systems in use for flow cytometry, including various formats of sheath flow injection. Because of considerations for solvent loading of the ICP (typically optimum for 25 to 80 μL/min), the "flow in air" (or in the instance of the ICP, "flow in argon") injector **171** may in some circumstances be considered most appropriate (though some improvement over current designs may be preferred, in order to minimize cell agglomeration). All sample introduction devices suitable for the purposes disclosed herein; including ICP devices, will serve, regardless of whether they now exist or are hereafter developed or improved.

For the feasibility experiments that we report below, a small volume spray chamber (similar in concept to a design reported by J. L Todoli and J. M Mermet, J. Anal. At. Spectrometry 2002, V17, 345-351) was employed, having a drain to remove condensed liquid (of which there was essentially none at the suspension flow rates used) and having no gas outlet except into the ICP.

It is noted that, compared to the FACS method for which careful alignment of the particles with the excitation laser is important, the present method allows relaxation of the alignment of the particles with the vaporizer, atomizer and ionizer (unless light scattering is used as the particle detection trigger; see later). This is because, especially for the ICP instance, the precise position of the particle within the injector tube feeding the ICP is of little importance to the detected signal (in part because the central channel flow containing the particle expands dramatically upon heating and in part because virtually all of the central channel flow is inhaled into the sampler of the ICP-MS vacuum interface, though only the predominantly central portion is subsequently transmitted through the skimmer; in any event, there appears to be substantial mixing of the central channel flow prior to sampling into the vacuum interface).

It is desirable that the entire particle introduced to the ICP be vaporized, and at least partially atomized and ionized, so as to enable determination of the element tags contained within the particle (intracellular tags, or bead labels). Current wisdom holds that solid particles (e.g., of glasses or geological materials) smaller than about 1 μm diameter, and liquid aerosols smaller than about 10 μm diameter, are efficiently vaporized, atomized and ionized in the ICP, while larger particles may be only partially volatilized. This is presumably due to the short transit time of the particle through the ICP, for which the heat transfer to a large particle is insufficient to allow complete vaporization, atomization and ionization. Thus, it is propitious to use beads having a diameter smaller than about 1 μm diameter (for example, we used stober silica particles of about 150 nm diameter in our feasibility studies described below). However, cells are frequently larger than 10 μm diameter. Nonetheless, our feasibility experiments, described below, suggest that cells larger than this perceived minimum are, in fact, efficiently vaporized, atomized and ionized, from which we infer that, upon the rapid heating during transit through the ICP, the cell explodes into fragments that are small enough to be vaporized, atomized and ionized. It remains possible that in certain instances the particles may be too large to allow efficient vaporization, atomization and ionization, which could be indicated by the failure to observe an intracellular tag or the element labels of a bead. In this instance, several ion source parameters (gas flow, power, sampling depth) can be adjusted to alleviate this deficiency. Alternatively, an in-line lysis component can be employed.

In-line Lysis

In-line lysis system **110** may be advantageously employed in some circumstances. For example, in the event that whole cell introduction is not viable, use of an in-line lysis system can be advantageous. This may be done by any method suitable for the purposes disclosed herein, including a number of methods now known to persons skilled in the art, including acidification of the sheath flow fluid to cause cell collapse or high purity (low conductivity) water sheath flow to induce rupture of the cell by osmotic pressure. In this instance, the elemental tags will be retained and transmitted to the device to vaporize, atomize and ionize the sample, though the transient pulse may be broadened slightly by diffusion in the flow stream.

Means for Vaporizing/Atomizing/Ionizing

Any means **104** suitable for the purposes disclosed herein can be employed to vaporize, atomize and excite or ionize the particle or the elemental tag associated with the particle; for example, graphite furnace, glow discharge and capacitively coupled plasma. Preferably, the vaporizer/atomizer/ionizer is an inductively coupled plasma. In some instances, vaporization, atomization and ionization and/or excitation can occur in different devices and at different times (e.g., within a graphite furnace for vaporization in combination with ICP for atomization and ionization and/or excitation.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) is a preferred means of determining the elemental composition, especially ultra-trace components, of materials. It has found acceptance in various applications including environmental (e.g., drinking, river, sea and waste water analyses), geological (e.g., trace element patterning), clinical (e.g., determination of trace metals in blood, serum and urine) and high purity materials (e.g., semiconductor reagents and components) analysis.

ICP-MS couples an inductively coupled plasma ionization source to a mass spectrometer. Briefly, a sample, most commonly an aerosol produced by nebulization, is injected into a high temperature atmospheric pressure plasma obtained by the coupling of radio frequency (rf) energy into the flowing argon gas stream. The resultant plasma is characterized by a high temperature (ca. 5000K) and relatively high concentration (ca. 1015 $cm.^{-3}$) of equal numbers of electrons and positive ions. Provided that the particles of the nebulized sample are small enough, as described above, the sample is promptly vaporized, atomized and ionized as it flows through the plasma. The efficiency of ionization is inversely and exponentially dependent on the ionization potential of the elements, with the majority of the periodic table being nearly 100% ionized. The resultant plasma containing the ionized sample components is extracted into vacuum where the ions are separated from neutral species and subjected to mass analysis. The "mass fingerprint" identifies the elements contained in the sample. The detected signal is directly and quantitatively proportional to the concentration of the elemental composition of the sample. The particular attributes of the method of note include: wide linear dynamic range (9 orders of magnitude), exceptional sensitivity (sub-part per trillion, or attomole/microliter, detection), high abundance sensitivity ($<10^{-6}$ overlap between adjacent isotopes for quadrupole analyzers), counting-statistics-limited precision, absolute quantification, and tolerance of concomitant matrix.

ICP-OES is another preferred method of performing the analyses described above; it is of particular merit when the solids content of the sample is greater than about 1% (for homogeneous liquid introduction rate of the order of 1 mL/minute). The conditions employed in the ICP are comparable to those described for the ICP-MS method. Detection of the emission from excited neutral atoms and ions in the ICP provides for the quantitative determination of the elemental composition of the sample. Most current ICP-OES instruments provide array detection for true simultaneous determination across most of the periodic table. In many favorable instances, ICP-OES retains some of the desirable characteristics of ICP-MS, including wide dynamic range and well-resolved detection channels. In other instances, there is potential for inter-element or molecular emission interference, though in such instances alternate emission wavelengths are frequently available. The principal deficiencies for the application considered here are its generally lower sensitivity (in some instances limited by background emission signals) and its inability to distinguish isotopes of a given element. Nonetheless, ICP-OES is perceived to be more simple to use, more robust, and less expensive than ICP-MS, and hence may have application for the present method.

Ion Pretreatment Device

In some circumstances, as for example in MS FC, an ion-pretreatment device **112** may be used to condition the ions for the mass analyzer. Because the mass spectrometer operates at reduced pressure (typically less than 10^{-4} torr) and the ion sources noted above typically operate at higher pressure (e.g., atmospheric pressure for the conventional ICP), one function of the ion-pretreatment device is to efficiently transport the ions derived from the sample through a pressure reduction step (the vacuum interface). It is desirable in this step, and subsequently, to increase the ratio of ions to neutrals that are subsequently transmitted to the mass analyzer. Ion optical components (ion lenses) typically serve this function, by localizing the ions and allowing the neutrals to be removed through vacuum pumps. An additional function of the ion optics is to condition the ion beam (in space and energy) to match the acceptance of the mass analyzer.

High-pass filter **116** and 'cooler' cells **118** are only two of the many suitable forms of pretreatment that now exist; doubtless other forms will hereafter be developed. Any devices or methods suitable for the purposes herein will serve.

Due to the short residence time of a single particle passing through the plasma, two separate ion handling (pretreatment) and mass separating techniques may be used.

A gain of two orders of magnitude relative to current ICP-TOF-MS instruments, which means about one order of magnitude greater than current quadrupole systems is also desired. The mass spectrometer-based flow cytometer is ideal for the detection of heavy atom tags. It is sufficient to determine only the mass range above ca. 100 amu. One of the most significant impediments to improved sensitivity is space charge repulsion of the dominant Ar^+ ions (m/z=40). Since the method is not limited by the conventional elemental analysis demands (the mass range of the typical elemental analyzer is from m/z=4 to m/z=250), it is possible to optimize the ion optics for the transmission of high mass ions.

While a conventional ICP-MS having simultaneous detection capability (for example, an ICP-TOF-MS **126** or ICP-ion trap-MS) is as a detector of the MS FC **101**, it should be realized that the requirements of the MS FC **101** are quite distinct from those of the conventional elemental analysis application. In particular, in the MS FC application the elements to be determined (as tags or labels) can be selected with advantage to be those above, say, 90 atomic mass units (amu, dalton, Thomson). In such instance, there is no need to provide simultaneously optimum sensitivity for low mass (e.g., Li, B, Na, Mg, Co, etc.) and high mass (e.g., the lanthanides and noble metals).

One approach employing a TOF analyzer **126** is to accelerate the ion beam relatively early in the plasma expansion because an accelerated beam has a higher space-charge-limited ion current and to high-pass filter the beam. This can be through the use of a quadrupole-type device, which is not pressurized. The depleted ion beam can then be decelerated (even collisionally cooled in a pressurized multipole, which could potentially also provide ion-bunching) prior to injection into the TOF. It is anticipated that the space-charge limit of such a continuous extraction beam is

sufficiently high to allow a ten-fold improvement in sensitivity for the higher mass ions.

An alternate, or concomitant approach, is to pulse-extract the ion beam. Since lower mass ions are accelerated to high velocity in a given extraction field, the Ar^+ ions (and lower mass ions, which can be discarded) run ahead of the higher mass ions of interest. Preferably, the front-running ions can be discarded using an orthogonal pulse, similar to "Smart-Gate" of the GBC TOF, (see, e.g., WO 98/33203) but in the ion optics region. The transmission window does not need to be precisely defined in this instance, as it is sufficient to intercept ions <100 amu. A downstream cooling cell could still be used to bunch the ions and normalize their energies. If the orthogonal pulser is problematic, the entire pulse-extracted ion beam can be run into the TOF extraction region, with the deficiency that more-narrow mass windows will be simultaneously injected into the TOF. Simple calculations (which overestimate the potential by at least some margin) indicate that a 15% duty cycle pulse-extractor could yield up to 28-fold (m/z=100) and 12-fold (m/z=238) sensitivity improvement over current (80 Mcps/ppm) quad systems. This assumes 100% transmission efficiency through the ion optics and 100% duty cycle of the TOF (requiring bunching).

The ion pretreatment device may also include a particle event trigger, which triggers instrument mass selection and detection systems to acquire data from discrete particles, and keeps the instrument idle between events. As is known to those skilled in the art, this can be done in many different ways.

Therefore, the ion pretreatment device may comprise:

a vacuum interface;

a high-pass mass filter downstream of the vacuum interface; and

a gas filled ion cooler cell downstream of the vacuum interface.

Among the distinctions that simplify the design of the MS FC **101** according to the invention relative to a conventional elemental analyzer are the relative invariance of the sample (cells or beads in a known buffer) that simplify the need for an ionizer design (e.g., ICP) that is tolerant of various sample types and matrices, the relative (with respect to the total ion current of the ICP) invariance of the total elemental composition of the sample that relieves the need to provide compensation for inter-element matrix suppression effects (recognizing that, for example, Na and Ca will be significant components of cells), and to a large extent (depending on the selection of tag elements) the need to compensate for the presence of spectral interferences due to argides, oxides and doubly charged ions. Thus, MS FCs according to the invention can be advantageously adapted to suit the cytometric application but not for the general elemental analytical application because of the selectability of the elements to be determined. For example, conventional elemental analysis by ICP-MS is compromised by the mutual repulsion of ions following extraction into the vacuum system; this space charge effect, well known to those skilled in the art, derives principally from the overwhelmingly large flux of lower mass ions that derive from the plasma support gas or the sample solvent such as O^+, Ar^+, ArO^+, $Ar2^+$, and in some instances lower mass ions that derive from other sample matrix components such as Na^+, Ca^+, Cl^+. It will be recognized that the most significant of these ions that form the bulk of the space charge effect are low mass ions, being below about 80 amu. Thus, advantage is to be had by eliminating such low mass ions as early as possible following extraction into the vacuum system because doing so will alleviate the space charge and its associated effective potential field barrier that suppresses transmission of other ions. Several schemes for achieving this relief can be conceived, including the use of a high-pass mass filter such as a quadrupole device that is operated to transmit ions above, say 80 amu. Notably, a quadrupole can be operated at the pressures extant in the ion optics region (typically about 10^{-3} torr). An additional advantage of such an ion pretreatment device for the present application is that it can also be operated to simultaneously provide a low pass mass filter function (that is, a bandpass between a selected low mass and a selected high mass). In the instance that a time-of-flight mass analyzer is used, this bandpass can provide an improvement in duty cycle (resulting in improved sensitivity) because it minimizes the incursion of the arrival of high mass ions from a previous pulse into the arrival time distribution of the current pulse and also the incursion of low mass ions from the previous pulse into the arrival time distribution of the current pulse (where "pulse" means the packet of ions that are injected into the flight tube of the time of flight mass analyzer). Further, acceleration of the ions as soon as possible upon their entrance to the vacuum system (or near the point where the debye length of the plasma is comparable to the dimensions of the apparatus or lenses) can further mitigate the space charge effects. However, in the instance that the ions are subsequently decelerated (for example, in the acceleration region for the TOF), the space charge effects can return and reassert themselves resulting in reduction of sensitivity and, in the instance of the TOF, reduced mass resolution due to energy broadening in the direction of the flight tube. Hence, the high pass mass filter, which can be functional at relatively high ion kinetic energy if appropriately designed, can be operated in concert with acceleration optics to mitigate space charge effects both immediately downstream of the vacuum interface and further downstream, for example in the acceleration region of a TOF mass analyzer **126**.

It is further advantageous, as is well known to those skilled in the art, that reduction of the axial ion energy by collisions with a non-reactive buffer gas in a pressurized multipole cell (a "cooler" cell) **118** provides improved resolution and sensitivity for TOF mass analysis (also expected to be true for an array-detector magnetic sector mass analyzer). Here again, the high pass mass filter **116**, which should precede the "cooler" cell **118**, can be operated in concert with the "cooler" cell **118** with advantage, since bandpassing the ions prior to the "cooler" cell **118** will mitigate to large extent space charge effects that otherwise would be detrimental (i.e., cause loss of sensitivity) in the "cooler" cell (which would happen because the ions are slowed by collisions in the "cooler" cell, and slowing them without first removing the bulk of the low mass "space charge inducing" ions causes an abrupt appearance of a significant defocusing space charge field near the entrance of the "cooler" cell).

As is known to those skilled in the art, in certain instances advantage is also to be had in including reactive gases in the "cooler" cell **118** in order to transform ions that are isobaric and thus are interfering or are interfered (reference U.S. Pat. No. 6,140,638). Further, the "cooler" cell can also be operated in a trap-and-pulse mode that could be optimized for synchronous operation with a TOF acceleration pulse to provide improved duty cycle (and hence sensitivity) for that mass analyzer. Thus, the MS FC **106** can incorporate with advantage ion acceleration optics and a high pass mass filter.

For several mass analyzer embodiments, including in particular the TOF and array-detector magnetic sector con-

figuration, the use of a gas-filled "cooler" cell is also advantageous. For the TOF configuration in particular, the high pass mass filter could with advantage be operated as a bandpass mass filter with both a low and a high mass transmission limit. As is known to those skilled in the art, the high pass mass filter and "cooler" cell can be combined as a single unit (cf., U.S. Pat. No. 6,140,638).

Advantage is also to be had, to minimize the volume of data collected to include only the most significant data or, in the instance of a mass analyzer (such as TOF) which is constrained by a duty cycle, to coordinate the measurement of data with the passage of a particle of interest through the detector system. In the conventional FACS method, this coordination is accomplished most often by the measurement of light scattering as the particle passes through the excitation region; the nature of this light scattering (forward and side light scatter) can provide information on the size and granularity of the particle which also has diagnostic value. In the MS FC or OES FC method, light scattering can be similarly used.

Where the source of excitation is an ICP, the scattering event can be detected prior to vaporization of the particle; hence a delay corresponding to the time or spatial delay required for signal generation. For OES FC this is the time or distance required for vaporization, atomization, ionization and emission; for MS FC an additional delay corresponding to the transit time of the extracted ions from the region of ionization to the mass analyzer is required. Those skilled in the art will realize that for continuous monitoring mass spectrometers, for example an array detector magnetic sector mass analyzer, this delay should be applied to the arrival of the ions at the array detector. For other mass analyzers, for example TOF and ion traps, the delay is applied to the device that introduces the ions into the mass analyzer, for example the acceleration region preceding the flight tube of the TOF or a pulsing lens that introduces ions to an ion trap, to which the subsequent mass analysis and detection is synchronized.

Other methods of providing a trigger for data collection are contemplated for the MS FC **106**. For example, it is expected that the passage of a particle through the ionizer (for example, the ICP) will cause an abrupt and consequent change in the mass distribution of the major ions that are extracted (for example, the dominant Ar^+ signal in ICP-MS could be suppressed with concomitant formation of C^+, H^+, Na^+, Ca^+, etc.). It is thus expected that the ion current ejected or the spatial position of this ion current ejection (due to differences in the stability characteristics of ions of different masses) from, for example, the high pass mass filter, will change significantly and can be detected with one or more electrodes within or external to, for example, the high pass mass filter. Further, the magnitude or duration of the current change detected may be correlated with the size or content of the particle and could provide further diagnostic information.

Other trigger devices are contemplated, including, for example, a detector that measures changes in the ion current or impedance or magnetic field associated with the ion beam extracted into the vacuum system.

Optionally, various components, including for example a high mass filter and a gas-filled ion cooler, may be provided in a single housing. This can provide, for example, improved durability, as well as improved operating, handling and installing qualities.

Mass Spectrometer

The pretreated ion cloud may be analyzed with a simultaneous mass analyzer. Sequential mass analysis (e.g., through the use of quadrupole devices) is also possible. Examples of simultaneous mass analyzers include TOF, 3D trap and Linear Trap.

In some instances where the MS FC **101** method is to be used to best advantage (e.g., multiplex assay of individual particles), a simultaneous mass analyzer is preferred. For example, in the instance of the use of an ICP as the vaporizer, atomizer and ionizer, the transient signals from a single particle may last for a period in the range 20 to 200 microseconds, which can be insufficient to allow quantitative multiplex assay using a sequential mass analyzer, for example a quadrupole mass analyzer. In such instances, examples of preferred mass analyzers include TOF, array-detector magnetic sector, 3D ion trap and linear ion trap. In other instances where the period of the transient signal is significantly longer, either by the nature of the device to vaporize, atomize and ionize or by broadening of the transient signal, for example through transport of the vaporized particles, atoms or ions through a length of tubing or through collisional processes (such as those reported by D. R. Bandura, V. I. Baranov and S. D. Tanner in J. Anal. At. Spect. 2000, V15, 021-928), a sequential mass analyzer may find utility.

At the current state of development of mass analyzers, the TOF appears to be best-suited for the MS FC application. Ion traps (3d and linear) might be suitable provided that they are preceded by a selection device, for example a high pass mass filter, that reduces the space charge in the trap. The array-detector magnetic sector analyzer, which offers high duty cycle and should provide high sensitivity, could be suitable provided that an efficient array detector is developed, though at the present state of development the abundance sensitivity (overlap of signals onto neighbouring mass channels) is limiting.

The most commonly-used mass analyzer **106** coupled to the ICP is at present the quadrupole, principally because of its robustness, ease of use, and moderate cost. However, the quadrupole is a sequential scanning analyzer having a cycle time for multiplex analysis that is long relative to the duration of a transient signal from a single particle in the plasma source. Therefore, the quadrupole cannot deliver correlated multi-analyte signals for such a short transient. A quadrupole ICP-MS analyzer is often used for the analysis of samples presented in quasi-continuous flow, for example for nebulization and laser ablation. It is appropriate for the analysis of homogeneous samples, such as for many conventional immunoassays where total element signaling is of interest.

In contrast, the time-of-flight (TOF) analyzer **126** shown in FIG. **2**, which samples a packet of ions in a given time period and spreads them in time according to their velocities in a potential field which are a function of the mass-to-charge ratios of the ions, is a "simultaneous" analyzer that is suited to the analysis of short transients such as those produced by single particles. Although TOF analyzers are known, the inventors are unaware of any TOF or other mass spectrometer analyzer currently being used for flow cytometry. Commercial ICP-TOF-MS instruments are some 10-100 times less sensitive than quadrupoles, at least in part due to more significant space charge effects in the ion optics and TOF acceleration region and to inefficiencies in duty cycle. With the employment of appropriate ion optics and other concepts noted herein, these deficiencies should be alleviated.

Another useful cytometer configuration is the OES FC **151** shown in FIG. **3**. A distinction between the OES FC **151** and the MS FC **101** is that in the former, light emitted by

both atoms and ions derived from the vaporized particle are collected and transmitted to an optical spectrometer having an array detector. In the ICP embodiment of OES FC **151**, the emission may be collected either radially through the ICP at a specified "height" above the rf load coil (the preferred observation height is a function of the plasma conditions, but is stable for stable ICP conditions) or axially by looking "down" through the plasma towards the injector (which requires a cooled viewing interface usually with a curtain flow of gas), as shown in FIG. **3**. The configuration and use of radial- and axial-viewed ICP-OES instruments is well known by those skilled in the art.

Among the distinctions of cytometers according to the invention from conventional fluorescence-based flow cytometry are that: (1) the cells or beads or analytes are tagged with elements rather than fluorophores; (2) the cells or beads are vaporized, atomized and (optionally, but usually naturally under optimum conditions) ionized and it is the elemental components of the cells and beads that are detected; (3) excitation to induce emission is gained from the ICP (convective and/or electron impact heating) rather than laser excitation at an absorption band of the fluorophore; (4) almost all elements of the periodic table are excited to emission (either atomic or ionic) under the operating conditions of the ICP, whereas multiple fluorophore excitation in conventional flow cytometry generally requires two or more excitation lasers, each of which may excite one or more fluorophores with absorption bands that are coincident with the wavelength of the excitation laser; (5) the emitted light is dispersed by, for example, eschelle gratings or prisms in one or preferably two dimensions and collected on an array of detectors, for example a CCD "camera", whereas the conventional flow cytometer uses bandpass optics to select a "least interfered" wavelength for each fluorophore; and (6) the emission wavelengths are more narrow in ICP-OES than in fluorescence-based flow cytometry, and there is usually more than one usable and detectable wavelength so that inter-element interferences are both less common (better resolved emission spectra) and more easily circumvented (by choosing an alternate emission wavelength).

EXAMPLES

Example 1—Development of Aptamers for Specific Labeling Leukemic Stem Cells

Leukemic stem cells and their progenitor cells can be purified [10]. They can be used as targets for selection of aptamers by selecting for the stem cell and against the progenitor cells using a novel method of combinatorial screening. The selected aptamers can be tested for, and selected against cross-reactivity with other aptamers directed for the multiplex assay of the challenge. The aptamers can be labeled with distinguishable stable isotopic elemental tags as is known to those skilled in the art.

Example 2—Preparation of Labeled MO7E Cell Line

A homogeneous MO7E cell-line which has been transduced with the p210 bcr/abl tyrosine kinase fusion protein from chronic myeloid leukemia can be used. This cell expresses the CD33 surface marker as well contains large amounts of p210 internally. The markers can be tagged with antibodies or aptamers suitably tagged with commercially-available tagging kits (NanoGold™, DELFIA™). The tagged affinity products can be incubated with fixed, permeabilized cells.

Example 3—Preparation of Quadrupole ICP-MS-Based Flow Cytometer

Demonstration of concept can be achieved using a quadrupole-based ICP-MS and the tagged cells of Example 2.

A flow cell can be constructed based on a direct injection nebulizer or a sheath-flow non-ionizing nanosprayer. A commercial flow cytometer can be used, but with modifications, and excluding parts related to fluorescence.

Single ion monitoring at the mass/charge of one of the tag elements has improved duty cycle relative to scanning mode so that many of the cell events are, observed. Subsequent measurement, in the same sample but at a later time, at the second surface tag element mass/charge will confirm independence of the affinity chemistry and detection, with the implication that simultaneous determination with an appropriate (TOF) detector is possible. Observation of the internal protein marker will provide important evidence that cell volatilization is achievable. If the internal marker is not detectable, in-line lysis can be used.

Example 4—Development of a Prototype Single Particle Injector

Referring to the injector **171** shown in FIG. **4**, the injector is used to inject cells (or beads or other particles) **400** together with the buffer solution into the desolvation chamber **403** surrounded by a heater **405**. The buffer solution flow is nebulized by high-pressure gas. The volatile component of the buffer and cells (mostly water) is transferred from aerosol to gas phase during the desolvation process and is expelled out of the desolvation chamber together with most of the nebulizer gas through exhaust vents **407**. In most cases, the nebulizer gas flow is limited by size and design of the injector (nebulizer). Therefore, some makeup gas can be introduced to allow complete desolvation. Desolvated heavy cells (or beads) escape directly into the straight cylindrical channel **409** with the rest of the gas and are introduced into vaporizer/atomizer/ionizer **104** of the EFC. In an embodiment the vaporizer/atomizer/ionizer is the ICP plasma, which allows .~1 l/min of gas to be introduced. Therefore, by adjusting the gap **411** between the desolvation chamber and the cylindrical channel housing, one can control desolvation as well as flow into the ICP plasma.

Example 5—Preparation of ICP-TOF-MS-Based Flow Cytometer Research Prototype Instrument

ICP-TOF-MS instruments are commercially available. The TOF mass spectrometer provides a simultaneous analyzer which is beneficial for mulitvariant analysis, of for example, rare leukemic stem cells.

An ICP-TOF-MS can be outfitted with a flow cell. The components of the instrument as shown in FIGS. **1**, **2**, and **3** and described in the preferred embodiment can be assembled. Relevant components of commercial products (ELAN® ICP-MS and prOTO® orthogonal MALDI-TOF) can be procured as the basis of a working system. Some modification of the operating system will be required to address the specific data collection issues of the cytometer prototype; suitable modifications are well understood by those of ordinary skill in the art. It can be sufficient to operate independent computer control systems for the ICP

source and the TOF analyzer, as this would allow rapid and efficient research investigation.

An instrument can be evaluated with respect to its analytical performance for homogeneous aqueous sample introduction as well as homogeneous cell digests. The ICP-TOF MS-based flow cytometer can be tested, for example, using human established leukemia cell lines (MO7e, K562, HL-60) to investigate the capabilities with respect to the needs for the cytometric application. Specifications for dynamic range, abundance sensitivity, transient signal pulse width and detection mode (analog/digital) for the research prototype instrument can be established.

The following examples have been demonstrated using a conventional quadrupole ICP-MS instrument (sequential scanning) using conventional nebulization of solutions obtained by acidification with HCl of the sample following immunoprecipitation and washing, which digested the sample yielding a relatively homogeneous solution. Thus, "simultaneous" determination refers in this instance to simultaneous immunoprecipitation followed by sequential measurement of the concomitant tags by ICP-MS.

Example 7—Dynamic Range of Anti-Flag M2 Agarose Bead Element-Tagged Immunoassay

FIG. **5** is a calibration curve of the ICP-MS linked immunoprecipitation assay of 3.times.FLAG-BAP. M2 agarose beads were used to capture samples of serially diluted 3.times.FLAG-BAP over a concentration range of 0.05 ng to 1500 ng per 100 μl 3×FLAG-BAP was detected using an anti-BAP primary antibody and an anti-mouse-nanoAu secondary antibody. Diluted HCl was used to dissolve the nanogold tag for ICP-MS sampling. The results indicate that the detected signal (for gold) is linearly proportional to the antigen (FLAG-BAP) concentration, and that at least 4.5 orders of magnitude of linear dynamic range are achievable. Large dynamic range is important in the cytometric application to permit simultaneous determination of biomarkers that appear in largely different copy-counts per cell or bead.

Example 8—Simultaneous Assay of Two Cytokines Using Beads

Fluorokine™ beads coupled with cytokine capture antibodies against either TNF-α. or IL-6 were mixed and exposed to a mixture of cytokines, including TNF-α. and IL-6, incubated and then probed with cytokine-specific antibodies tagged with Eu (for anti-TNF-α.) and Tb (for anti-IL-6). After washing and digestion with HCl, the solution was analyzed for Eu and Tb. FIG. **6** provides calibration curves derived from this simultaneous immunoassay experiment. Linearity of signal with antigen concentration over at least 3 orders of magnitude is observed.

Example 9—Simultaneous Assay of Two Proteins Using ICP-MS-Linked Maleylation Immunoassay

FIG. **7** shows the simultaneous quantitation of two proteins using a direct immunoassay conducted in a Reacti-bind Maleic Anhydride 96 well plate, coupled to ICP-MS detection. In this experiment, two proteins (Human IgG and 3×FLAG-BAP) in 1×PBS were incubated in triplicate for one hour at room temperature to allow binding to the surfaces of the well of the Maleic Anhydride plate. Negative controls consisted of 100 μl PBS without protein. The plate was probed with primary antibodies anti-Human Fab'-nanoAu and anti-FLAG-Eu, washed and acidified with 10% HCl with 1 ppb Ir and 1 ppb Ho as internal standards [11]. Homogeneous samples were used wherein the elemental tag(s) are released to acidic solution for conventional nebulizer introduction to the ICP-MS. Note that the sensitivity to IgG using the nanogold tag is approximately 10 times greater than that for FLAG-BAP using the Eu tag; this is because each nanogold tag contains approximately 70 gold atoms (Au is monoisotopic) while each Eu tag contained only between 6 and 10 Eu atoms, approximately equally distributed between the two natural isotopes of Eu (^{151}Eu and ^{153}Eu, the sum of which were measured). The example demonstrates that at least two proteins can be immunoreacted simultaneously and detected without mutual interference, and that the sensitivity scales with the concentration of the antigen and with the number of atoms of the measured isotope per tag.

Example 10—Preparation of a Kit For the Analysis of an Analyte Bound to a Single Cell by Mass Spectrometry

A kit is assembled comprising (1) a tagged biologically active material which binds to an analyte of interest bound to a single cell and (2) instructions for single cell analysis by mass spectrometry.

Example 11—Forensic Applications

The methods and apparatus of the present invention can be used for forensic applications. For example, the methods and apparatus can be used to:

- determine antigenic blood types (ABO and Lewis types);
- identify body fluid (blood, semen, saliva) and other biosamples (whole blood, plasma, serum, urine, cerebrospinal fluid, vitreous humor, liver or hair);
- determine tissue origin (species, personal identity, etc.);
- determine paternity.

Example 12—Transfusion Medicine

The methods and apparatus of the present invention can be used in transfusion medicine to:

- resolve blood group A, B and D typing discrepancies;
- determine the origin of the engrafted leukocytes in a stem cell recipient; and
- determine the origin of lymphocytes in a patient with graft-versus-disease.

Example 13—Flow Cytometer with ICP-MS Detector Feasibility Test

We have performed feasibility studies to validate the concept of the present invention. A quadrupole (sequential mass scanning) ICP-MS instrument designed for conventional elemental analysis (and thus not optimized for the flow cytometric application) was used. The instrument was modified in only two ways: a modified sample introduction system was installed, and an oscilloscope was attached in parallel with signal handling hardware and software of the original detector system.

The sample introduction system **102**, **800** is shown schematically in FIG. **8**. Sample **400** consisting of cells or other particles was aspirated using a syringe pump connected with capillary tubing **801** to a small volume spray chamber **803** having a drain **805** to remove condensed liquid and having no gas outlet except into the ICP through the 2 mm diameter injector tube **807**. Sample was pumped at 50 μL/min, about

half of which was drained from the spray chamber **803** and half delivered to the ICP. The inventors recognize that this sample introduction device **800** may not necessarily optimum for presentation of single particles to the ICP with high efficiency in all cases, depending upon the circumstances of the analysis, but it was sufficient in this case to introduce at least a fraction of the particles into the ICP and thus to show feasibility.

The discrete dynode detector of the ICP-MS instrument provides signals that are either analog or digital (pulse). The analog signal, taken part way along the dynode chain, is converted to digital output in the hardware and software of the detector system. The digital (pulse) signal is taken from the final dynode of the chain, is amplified and transient signals corresponding to single pulse events whose amplitudes exceed a given threshold are counted in the detector system hardware and software. The detector system hardware and software can be configured to provide output not of each pulse, but the integral of these over a specified measurement period (minimum about 100 microseconds). In normal operation, if the signal detected at the analog dynode exceeds a specified threshold, the dynode chain downstream is disabled (disabling digital signal detection). If the analog signal is higher than a second threshold, the detector firmware adjusts the voltage of the ion optics of the instrument to defocus the ions from the detector in order to protect the detector. An oscilloscope was tapped into the analog output and operated in parallel with the detector hardware to enable the measurement of the transient events over the period of a single particle event in the ICP (e.g., up to several milliseconds with as low as a few nanoseconds resolution).

Because the instrument used for these experiments is not capable of measurement of more than one mass/charge channel during a short transient period, multiplex analysis of a single particle event in the ICP was not be demonstrated. However, measurement of single mass/charge detection channel events has allowed demonstration and evaluation of certain important characteristics of the ICP-MS detector system for the cytometric application. The inventors believe that these characteristics can be replicated, with some differences depending on the selected embodiment of the instrument configuration, with a simultaneous mass analyzer, with the additional benefit of facilitation of simultaneous measurement of many mass/charge detection channels permitting multiplex assay of single particles.

Feasibility Test 1: Detection of Single Particle Events, and Estimate of Sensitivity of Current Instrument

The MO7e cell line is a human megakaryocytic leukemia-derived cell. MO7e expresses CD33 antigen (67 kDa single chain transmembrane glycoprotein, myeloid cell surface antigen CD33 precursor (gp67)). The cell is thought to express approximately 5000 to 10000 copies of antigen per cell. The cell line was used to demonstrate that individual cells can be observed by methods according to the invention, and to estimate the sensitivity of such method using the current instrumentation. The CD33 surface marker was detected using monoclonal anti-CD 33 (IgG1 mouse) and Nanogold™-tagged anti-mouse secondary antibody (approximately 70 Au-atoms per tag). It is estimated that the efficiency of secondary antibody staining is approximately 10%.

Materials

MO7e cells were cultured for three days in a T75 flask. The cell concentration was determined by hemocytometer and found to be $0.5.\times10^6$ cells/ml.

Monoclonal antibody anti-CD33, unconjugated. IgG1 (mouse) isotype supplied at 2 mg/ml and purified in PBS/BSA with 0.1% sodium azide by Immunotech Inc. Cat#1134.

Secondary anti-mouse IgG conjugated with nanogold from Nanogold Inc. (approximately 70 Au-atoms per tag)

1% formalin prepared from 37% formalin; diluted in PBS.

Wash and antibody dilution buffer PBS/1% BSA.

50 mM ammonium bicarbonate buffer, pH 8.0.

Procedure

Tubes were soaked in PBS/1% BSA for one hour. MO7e cells were pelleted at 1500 rpm (at approximately 200 g) 5 min, resuspended in 5 ml PBS, pelleted and the wash discarded.

Cell pellet was resuspended in 3 ml PBS/1% BSA and distributed into three eppendorf tubes (at approximately 106 cells/tube) marked as primary and secondary antibodies added; only secondary antibody added; or no antibodies added.

Primary antibody was diluted 1:50 in PBS/1% BSA and added to the cell pellet for 30 min on ice.

Cells were washed with PBS/1% BSA once.

Secondary antibody was diluted 1:50 in PBS/1% BSA and added to cell pellet for 30 min on ice.

Cells were washed once with PBS/1% BSA, once with PBS.

Live stained cells were fixed in 1% formalin/PBS for 10 min RT and left in the fixative on ice overnight.

Cells that did not receive antibodies were treated only with PBS/1% BSA concordantly with the stained cells.

Stained formalin fixed cells were pelleted at 1500 rpm (at approximately 200 g) for 5 minutes and resuspended in 1 ml 50 mM ammonium bicarbonate buffer, pH 8.0 per tube next day. This was discarded after centrifugation and fresh bicarbonate (0.5-1 ml) was added to each tube.

Tubes were vortexed gently to break up the pellet, left to sit for 5 minutes for large clumps to settle to the bottom, and the top 25 μL of whole cell suspension were injected into the ICP-MS instrument.

Observations

The integrated (pulse detector) signal for Au for discrete cell introduction gave 300-500 counts per second (cps), secondary antibodies only, less than 100 cps, no antibodies, less than 10 cps and buffer only, less than 3 cps.

FIG. **9** shows the overlaid results of separate direct injections of the 100 ppt Rh (1% HNO3) and cell suspension (separate injection, 50 mM NH4HCO3) samples as described above.

FIGS. **10**A and **10**B show oscilloscope data associated with FIG. **9**. FIG. **10**A (on the left) shows the signal for Ar_2^+ (about 10^7 cps) signal. The upper trace covers a relatively large time window (ca. 100 .μs), from which one could conceivably determine the average ion signal rate. The lower trace shows the pulse for a single ion detection event (over a greatly magnified time scale). FIG. **10**B (on the right) shows Au^+ from cell introduction. The upper trace indicates that multiple ion signal pulses are not observed. The lower trace shows the signal pulse for a single Au ion detection event. Typically, only one ion pulse was observed in a particle event time window, suggesting that we detect on average only about one Au atom per cell.

Efficiency of Detection

We estimate the sample introduction rate at (very) approximately 400 cells/second, derived as follows: approximately 1×10^6 cells per sample, 1 mL/sample at 50 .μL/minute introduction sampling with 25 .μL/min delivered to the ICP.

Accordingly, we infer that approximately one Au atom per cell is observed.

The detection efficiency of the instrument used was estimated from the signal obtained from continuous aspiration of a sample containing 100 parts per trillion (ppt, mass/volume) in 1% nitric acid. The signal obtained was approximately 2000 cps, suggesting an efficiency for detection of Rh of 1×10^{-5}, derived as: 100 ppt (10^{-10} g/mL), atomic weight 103 g/mole, 25 μL/min delivered to plasma, yielding 2×10^{8} atoms Rh/second delivered to plasma for which 2000 cps is observed. The inventors ascribe this efficiency to the following: 100% ionization, 1% transmission through the vacuum interface (100% of central plasma containing ion inhaled through sampler, 1% of sampler flow transmitted through skimmer), 10% transmission through mass analyzing quadrupole, and therefore about 1% transmission through the ion optics. (From these estimates, we infer that improvements in sensitivity for the cytometric application, assuming retention of the vacuum interface configuration, should principally focus on improving the transmission through the mass analyzer (e.g., TOF with high duty cycle) and, more importantly, the ion optics (according to the earlier discussion, principally through accelerating optics and elimination of space-charge-inducing ions)).

Therefore, if one Au atom detection event per cell is obtained, and this is obtained with the same detection efficiency as Rh solution, we estimate that the MO7e cell averages approximately 1400 tagged CD33 markers per cell. With the assumption that the efficiency of the two antibody tagging is about 10%, the estimated number of CD33 per cell is 14000, which is consistent with the 5000-10000 quoted earlier. It is desirable to provide higher sensitivity so that proteins of lower copy-count per cell can be detected. In addition to the ion optical improvements suggested above, direct immuno-tagging (as opposed to the 2 antibody sandwich used here) is expected to be advantageous.

We conclude that the method is able to detect single particle events in the plasma. The experiments described provide guidance for research efforts to improve the sensitivity of the method. A simultaneous mass analyzer is required to facilitate the multiplex advantage that the mass spectrometer detector provides to flow cytometry.

Feasibility Test 2: Estimation of the Transient Period of a Single Particle Event

The MO7e cell sample used in Feasibility Test 1 provides an opportunity for the estimation of the transient period of a single particle event, which is important for the design and optimization of the MS FC. It is estimated that the NaCl content in the cell is 0.9% w/w. For a 16 micrometer cell this converts to 2×10^{11} atoms of Na per cell. The efficiency of the instrument used in these experiments for Na detection is lower than for Rh; about 1×10^{-6}. Thus, for a single cell event, 2×10^{5} ions will reach the detector. This is a sufficiently-large number that the arrival period of Na ions corresponding to a single MO7e cell event can be measured.

If the transient produced by the single cell event is of the order of 100-300 microseconds (as reported by Olesik for monodispersed 3-65 micrometer particles), an equivalent average count rate of $(0.7\text{-}2)\times10^{9}$ is achieved (with peak current about twice that).

FIGS. **11**A and **11**B show the Na^+ signal detected at the oscilloscope over the period of several cell introduction events. The data given in FIG. **11**A shows the Na^+ signal when cells are introduced in a 30 mM $CaCl_2$ buffer. The data shown in FIG. **11**B presents the results for buffer only. The variability of the observed signals may reflect the variability in cell size (volume and thus Na content) of the cell population, or might indicate the presence of Na-containing particles other than MO7e cells. The important observation, for the present purposes, however, is that the transient signal for a single particle event is of the order of 100-150 μs.

The baseline is very different between the two datasets shown in FIGS. **11**A and **11**B. This difference can be attributed to the fact that a first cell detected should trip the higher threshold detector protection circuitry and activate ion defocusing. This is because the anticipated 2×105 Na ions per cell arrive in the period of approximately 100-150 μs, which corresponds to an average count rate exceeding 109 per second, is sufficient to trip the second threshold detector. In the absence of cells in the sample (data given in FIG. **11**B), the detector protection circuitry is not tripped. The ion optical defocusing appears to suppress ion transport by about a factor of 1000, accounting for the difference in the baseline data, but this is not intended to be a stable or reproducible (quantitative) defocusing factor.

Transient signals of 100-150 μs period, ascribed to MO7e introduction events, were observed at a frequency of about 5 to 6 per 10 milliseconds, or about 500 to 600 cells per second. This is consistent with the estimates made earlier, and with the estimate of 106 cells per 1 mL in the original sample (procedure step 4 of Feasibility Test 1, subsequently reduced to approximately 1 mL volume in step 9).

A notable inference taken from this experiment is that the high Na^+ signal anticipated for cells, or effects related to the change in mass distribution of the plasma ions as a result of the passage of a particle through the plasma, might provide a means to trigger the system upon a cell event. Further, it is feasible that the magnitude of the Na^+ signal (or signal of another element at high concentration in the cell), or the magnitude of effects related to the ion distribution change as a result of the particle's passage through the plasma, could be correlated with the physical size of the particle, which may be of importance in identifying target particles or distinguishing single particles from groups of particles.

The important conclusion of this experiment is that the transient signal is approximately 100-150 μs FWHM in duration. This has implications for design considerations to provide dynamic range. Further, it is evident that particles characterized by transient signals of this period can be introduced to the system at a rate of about 3000 per second (so that signal corresponding to a particle is present up to 50% of the time). Smaller cells, and smaller beads, should have shorter transients, and thus allow higher rate of introduction.

Feasibility Test 3: Comparison of Current FACS With the Current ICP-MS With Cell Injection, and Demonstration of Entire Cell Volatilization

The inventors have had an opportunity to compare directly the performance of a current FACS instrument to that of the ICP-MS instrument with cell injection described in Feasibility Test 1. Further, the test was configured to provide for tagging of intracellular proteins; if these internal tags can be detected, this implies that the entire cell and its contents were vaporized, atomized and ionized, rather than just vaporization of surface tags.

Because the ICP-MS instrument used for these experiments was not a simultaneous detector, the same (nanogold) tags could be used for each antigen, and immuno-tagging was performed in separate vials for each sample and antigen. Thus, each antigen for each sample was determined in a separate analysis. Samples were introduced to the ICP-MS as described in Feasibility Test 1.

Preparation of Samples for ICP-MS Analysis with Cell Injection

Materials

Human Monocyte Cell Lines:

MO7e parent line is a human megakaryocytic leukemia-derived cell. MO7e express CD33 antigen (67 kDa single chain transmembrane glycoprotein, myeloid cell surface antigen CD33 precursor (gp67)). Approximately 5000-10000 copies of CD33 antigen per cell.

MBA-I and MBA-4 are stable clones of MO7e transfected with p210 BCR/Abl expression plasmid.

HL-60 (ATCC cat #CCL-240), myeloid leukemia cell line used as antigen in production of anti-CD33 monoclonal antibodies.

Antibodies:

anti-CD33, mouse monoclonal, unconjugated. IgG 1 (mouse) isotype. Supplied at 2 mg/ml purified in PBS/BSA with 0.1% sodium azide (Immunotech Inc. Cat#1134)

anti-IgG2a, mouse, (BD PharMingen, cat #555571) (0.5 mg/ml stock)

anti-BCR antibody raised in rabbit (Cell Signaling Tech. Cat#3902), used at 1:25 for flow cytometry

Secondary antibodies: 2001 nanogold-anti-mouse IgG (NMI) and 2004 nanogold anti-rabbit Fab' (NRF) (Nanoprobes Inc.) used at (1:50) according to manufacturer's recommendation.

Buffers:

BD Biosciences FACS permeabilization solution 2 (cat #347692) PBS with Ca++/Mg++;

PBS/1% BSA

1% and 0.5% formalin prepared from 37% formalin; diluted in PBS 50 mM ammonium bicarbonate buffer, pH 8.0

Procedure:

Tubes were soaked in PBS/1% BSA for one hour.

Cells were pelleted at 1500 rpm (~200 g) 5 min, resuspended in 5 ml PBS and counted using a hemocytometer. Cell yield:

MO7e-1e6/ml

MBA-1-1e6/ml

MBA-4-1e6/ml

HL-60-1e6/ml

MO7e (tube #1) and HL-60 (tube #2) were stained live with anti-CD33 (1:50) on ice for 30 min; followed by one wash with PBS/BSA. Anti-mouse-IgG-Au (1:50) was added to the washed cell pellet for another 30 min on ice. Live stained cells were fixed in 1% formalin/PBS for 10 min RT and left in the fixative on ice over 48 hours.

MBA-I (tube #3), MBA-4 (tube #4) and MO7e (tube #5) were permeabilised and fixed in the FACS Permeabilization Solution 2 for 10 min at RT.

After one wash the cells were incubated in media with 10% FBS to block non-specific antigen sites for 15 min RT.

Permeabilized cells were treated with anti-BCR antibodies (1:25) (tubes #3,4,5) or with non-specific IgG (tubes #3°., 4°., 5°. w/o primary antibody) for 45 min RT. Secondary antibodies were added to washed cells anti-rabbit-IgG-Au (1:50) for 45 min RT.

Stained cells were washed twice prior to post-fixation in 0.5% formalin and kept in fridge over the weekend prior to MS analysis when the formalin was replaced with 50 mM ammonium bicarbonate.

Preparation of Samples for FACS Analysis (Carried Out Simultaneously With Above)

Materials

Antibodies:

anti-IgG 1-FITC mouse isotype, (BD PharMingen)

anti-CD45-FITC antibody raised in mouse (BD PharMingen) used at 1:50 for flow cytometry. CD45 is expressed on the surface of all human leukocytes. Used as a positive sample for FACS set-up.

Secondary fluorescent antibodies: anti-mouse IgG-FITC (BD PharMingen) and anti-rabbit-FITC (Biolab) used at (1:50)

Buffers:

BD Biosciences FACS permeabilization solution 2 (cat #347692)

PBS with Ca++/Mg++;

PBS/1% BSA

1% and 0.5% formalin prepared from 37% formalin; diluted in PBS 50 mM ammonium bicarbonate buffer, pH 8.0

Procedure

Cell preparation and primary antibody staining was done in parallel with samples for ICP-MS with 1e6 cells/ml/tube.

All procedures with fluorescent secondary antibody staining and cell washes were carried out in the dark on ice.

After the final PBS wash cells were resuspended in PBS (not formalin) and immediately processed by FACS (BD FACSCalibur).

Gates and settings were determined using the anti-CD45-FITC stained HL-60 as positive (R4) channel and isotype anti-mouse IgG-FITC stained HL-60 as negative (R3) channel.

Observations

The results for both the ICP-MS detection (shaded grey) and conventional FACS (white) are summarized in FIG. **12**.

Standard deviations for triplicate analyses by ICP-MS are shown by error bars; equivalent uncertainties for the FACS results were not provided.

Both the CD33 (surface markers on MO7e and HL60) and the BCR (internal marker in MO7e, MBA1 and MBA4) were determined by both FACS and ICP-MS detectors. This implies that the entire (permeabilized) cells and their contents were vaporized, atomized and ionized in the ICP-MS. Further, the FACS and ICP-MS results are largely in rather good agreement for both the surface and internal markers and for the procedural blanks.

We conclude from these results that FACS and ICP-MS detection (using the current un-optimized instrument) provide comparable results for single antigen assay. It is anticipated that the sensitivity of the ICP-MS detector will be improved as discussed above, and that incorporation of a simultaneous mass analyzer will permit high order multiplex assay. It is also evident that the MO7e, MBA1 and MBA4 cells used in these experiments were efficiently vaporized, atomized and ionized. This suggests that the optional in-line lysis device discussed above is not required for these or similar cells.

Feasibility Test 4: Production and Detection of Element-tagged Beads

Another approach to multiplexed assay is to use different identifiable beads that immobilize antigens. The beads typically have capture affinity agents (e.g., antibodies) attached to their surface. After exposure to a sample, the bead-antigen complexes are typically exposed to a second affinity product (antibody, aptamer, etc.) which is tagged with an element or isotope as already discussed (herein, and in U.S. patent application Ser. No. 09/905,907, published under US 2002/

0086441 on Jul. 4, 2002 and Ser. No. 10/614,115). The beads are distinguished by their elemental composition, which might be a surface element label, and encapsulated element label or an element label incorporated within the bead material. The identity of the bead can be associated with the type of capture affinity agent attached to the bead or to the sample (e.g., beads with different element labels are exposed to different samples, or are placed in different wells of a 96- or 384- or 1536-well plate). Thus, detection of the secondary affinity product tag determines the presence of the antigen and the element composition (element label) of the bead indicates which antigen was captured or the sample in which it was captured. The method is modeled after U.S. Pat. No. 6,524,793, assigned to Luminex, and references therein.

The beads may be of any appropriate material (e.g., polystyrene, agarose, silica). Each bead may contain one or more affinity capture agents, and multiplexed assay of the antigens captured on the bead may be conducted. The element label incorporated in or on the bead may be a single element or isotope or, preferably, a combination of elements or isotopes. For example, if the dynamic range of the detector is three orders of magnitude and differences in signal levels of a factor of three are reliably detected, two element labels can be combined in different ratios to provide 63 distinguishable beads. Under the same conditions, 5 element labels can provide 32,767 distinguishable beads. With 5 orders of dynamic range and 5 element labels for which factors of three in signal can be reliably detected, 248,831 distinguishable beads can be constructed. It will be recognized that the beads can be manufactured to a size suitable for complete vaporization, atomization and ionization in the device used for that purpose (e.g., ICP). It will also be recognized that smaller beads are likely to provide shorter transient signals, and that accordingly the rate of particle introduction can be optimized for the particular beads used.

To demonstrate the viability of the method, stober silica particles having a diameter of about 150 nm were grown in various lanthanide (Ho, Tb, Tm) solutions. The lanthanide elements were incorporated into the silica particles. The silica particles (beads) were introduced serially to the ICP-MS instrument as described in Feasibility Test 1. Since the instrument used was not capable of simultaneous multielement analysis, the transient signals for the lanthanides and for silicon were measured separately for different beads.

FIGS. **13**A and **13**B show some of the data obtained. The data provided in FIG. **13**A shows the detection of Si^+, clearly indicating that the beads are vaporized, atomized and ionized. Data provided in FIG. **13**B show the detection of Tb^+ (for beads grown in Tb solution). Clearly, the Tb label is detected. If a mixture of beads having different lanthanide labels were sampled, the different lanthanide signals would identify the different beads. It is also evident that beads can be grown in solutions of mixed lanthanides (or other elements), and would incorporate the different elements, thus providing for a larger number of distinguishable beads as indicated above. The availability of a simultaneous analyzer would further allow simultaneous detection of the elements associated with the bead itself and also with the tag associated with a secondary affinity product that recognizes a captured antigen.

Therefore, elements within a bead can be detected (i.e., the bead is vaporized to its atomic components). Different combinations of element internal "labels" can be used to distinguish beads. If those beads carry different surface antibodies to bind different antigens, and those antigens are then recognized by another antibody containing a different element reporter tag, a multiplexed assay is enabled. Alternatively, the differently labeled beads can be used with the same surface antibodies, but with the different beads being applied to different samples (such as a 96 well plate), so that the signal associated with the labeled affinity product identifies the antigen concentration in the sample indicated by the signals corresponding to the bead composition. Numerous modifications, variations and adaptations may be made to the particular embodiments of the invention described above without departing from the scope of the invention, which is defined in the claims.

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What is claimed is:

1. A method of sequentially analyzing single cells by mass spectrometry, comprising:

providing a sample containing a plurality of tagged cells tagged with a plurality of tagged antibodies, wherein each of the tagged antibodies is specific for a different analyte, and wherein each of the tagged antibodies is tagged with an elemental tag comprising a lanthanide or noble metal;

vaporizing, atomizing, and ionizing multiple elemental tags from a single first cell of the plurality of tagged cells;

detecting, using mass spectrometry, the elemental composition of the first cell by detecting a transient signal

of the multiple vaporized, atomized, and ionized elemental tags of the first cell;

vaporizing, atomizing, and ionizing multiple elemental tags from a single second cell of the plurality of tagged cells; and

detecting, using mass spectrometry, the elemental composition of the second cell by detecting a transient signal of the multiple vaporized, atomized, and ionized elemental tags of the second cell, wherein the transient signal associated with the first cell and the transient signal associated with the second cell are detected sequentially.

2. The method of claim **1**, further comprising lysing the plurality of tagged cells prior to vaporizing, atomizing, and ionizing the multiple element tags from the first cell.

3. The method of claim **2**, wherein vaporizing, atomizing, and ionizing the multiple elemental tags from the first cell comprises sequentially vaporizing, atomizing, and ionizing fragments of the first cell.

4. The method of claim **1**, wherein detecting the elemental composition of the first cell comprises sequentially detecting fragments of the first cell.

5. The method of claim **1**, wherein at least one of the plurality of tagged antibodies is tagged using cyclic diethylenetriaminepentaacetic acid anhydride (cDTPA), isothiocyanatobenzyl-DTPA (SCN-Bz-DTPA), or 1,4,7,10-tetraazacyclododecanetetraacetic acid (DOTA).

6. The method of claim **1**, wherein at least one of the multiple elemental tags from the first cell comprises multiple copies of an element or isotope.

7. The method of claim **6**, wherein the at least one of the multiple elemental tags from the first cell comprises at least 70 atoms.

8. The method of claim **1**, wherein at least one of the multiple elemental tags from the first cell is a nanogold reagent.

9. The method of claim **1**, wherein each of the plurality of tagged antibodies is tagged with a distinct isotope.

10. The method of claim **1**, wherein detecting the elemental composition of the first cell comprises distinguishing transient signals from the multiple vaporized, atomized, and ionized elemental tags of the first cell.

11. The method of claim **1**, wherein vaporizing, atomizing, and ionizing the multiple elemental tags from the first cell comprises providing the multiple elemental tags of the first cell to an inductively coupled plasma torch.

12. The method of claim **1**, wherein vaporizing, atomizing, and ionizing the multiple elemental tags from the first cell comprises providing the multiple elemental tags of the first cell to a glow discharge device.

13. The method of claim **1**, further comprising pretreating the multiple vaporized, atomized, and ionized elemental tags of the first cell, wherein detecting the elemental composition of the first cell comprises detecting the multiple pretreated, vaporized, atomized, and ionized elemental tags of the first cell.

14. The method of claim **13**, wherein pretreating the multiple vaporized, atomized, and ionized elemental tags of the first cell comprises depleting an ion beam of the multiple vaporized, atomized, and ionized elemental tags of the first cell to reject ions at or below the atomic mass of Calcium.

15. The method of claim **14**, wherein depleting the ion beam comprises passing the ion beam through a high pass ion guide.

16. The method of claim **14**, wherein depleting the ion beam comprises passing the ion beam through a pulse-extractor.

17. The method of claim **14**, wherein detecting the elemental composition of the first cell comprises injecting the depleted ion beam into a time of flight mass spectrometer.

18. The method of claim **13**, wherein pretreating the multiple vaporized, atomized, and ionized elemental tags of the first cell occurs in a vacuum.

19. The method of claim **1**, wherein detecting the elemental composition of the first cell comprises detecting the multiple vaporized, atomized, and ionized elemental tags of the first cell by a time of flight mass spectrometer.

20. The method of claim **1**, wherein detecting the elemental composition of the first cell comprises detecting the multiple vaporized, atomized, and ionized elemental tags of the first cell by a magnetic sector mass spectrometer.

* * * * *

EXHIBIT B

US010072104B2

(12) **United States Patent**
Winnik et al.

(10) **Patent No.: US 10,072,104 B2**
(45) **Date of Patent: Sep. 11, 2018**

(54) **POLYMER BACKBONE ELEMENT TAGS**

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(73) Assignee: **Fluidigm Canada Inc.**, Markham (CA)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **14/875,553**

(22) Filed: **Oct. 5, 2015**

(65) **Prior Publication Data**

US 2016/0025731 A1 Jan. 28, 2016

Related U.S. Application Data

(62) Division of application No. 14/659,224, filed on Mar. 16, 2015, now Pat. No. 9,296,838, which is a division
(Continued)

(51) **Int. Cl.**
G01N 33/574 (2006.01)
C08F 220/56 (2006.01)
(Continued)

(52) **U.S. Cl.**
CPC ***C08F 8/32*** (2013.01); ***C08F 8/14*** (2013.01); ***C08F 8/42*** (2013.01); ***C08F 220/36*** (2013.01);
(Continued)

(58) **Field of Classification Search**
CPC .. C08F 8/42; C08F 220/36; C08F 8/32; C08F 220/56; C08F 8/14; G01N 33/532; G01N 33/58; G01N 33/57426; Y10T 436/24
See application file for complete search history.

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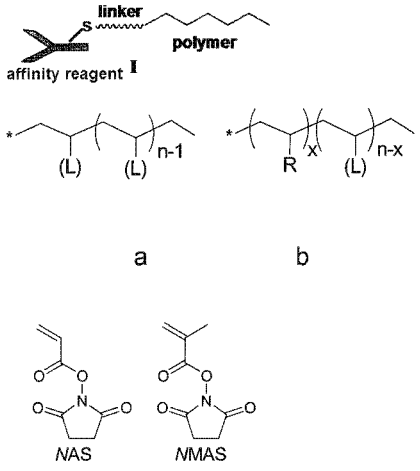
Primary Examiner — Shafiqul Haq
(74) *Attorney, Agent, or Firm* — Kilpatrick Townsend & Stockton LLP

(57) **ABSTRACT**

Element tags based on novel metal-polymer conjugates are provided for elemental analysis of analytes, including ICP-MS. A polymer backbone is functionalized to irreversibly bind metals that are selected prior to use by the user. The polymer is further functionalized to attach a linker which allows for attachment to antibodies or other affinity reagents. The polymer format allows attachment of many copies of a given isotope, which linearly improves sensitivity. The metal-polymer conjugate tags enable multiplexed assay in two formats: bulk assay, where the average biomarker distribution in the sample is diagnostic, and single cell format to distinguish a rare (for example a diseased) cell in a complex sample (for example, blood).

19 Claims, 16 Drawing Sheets



Related U.S. Application Data

of application No. 11/754,340, filed on May 28, 2007, now Pat. No. 9,012,239.

(60) Provisional application No. 60/803,356, filed on May 27, 2006.

(51) **Int. Cl.**

C08F 8/32	(2006.01)
C08F 8/42	(2006.01)
C08F 220/36	(2006.01)
G01N 33/532	(2006.01)
G01N 33/58	(2006.01)
C08F 8/14	(2006.01)

(52) **U.S. Cl.**

CPC ***C08F 220/56*** (2013.01); ***G01N 33/532*** (2013.01); ***G01N 33/57426*** (2013.01); ***G01N 33/58*** (2013.01); *Y10T 436/24* (2015.01)

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2015/0183895	A1	7/2015	Winnik et al.

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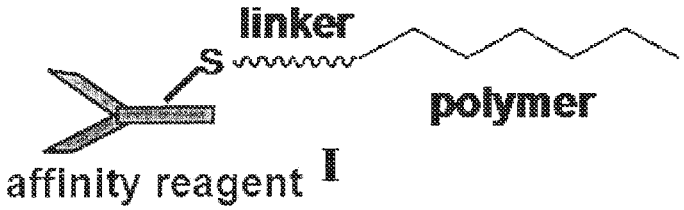
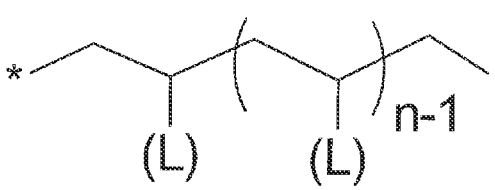
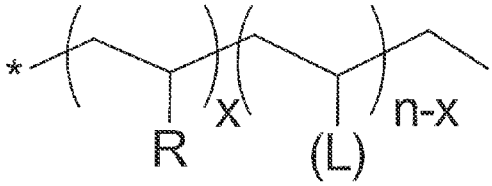
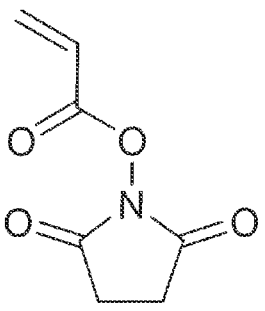
Figure 1



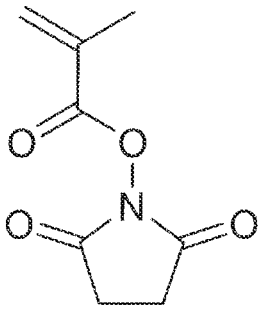




a b



NAS



NMAS

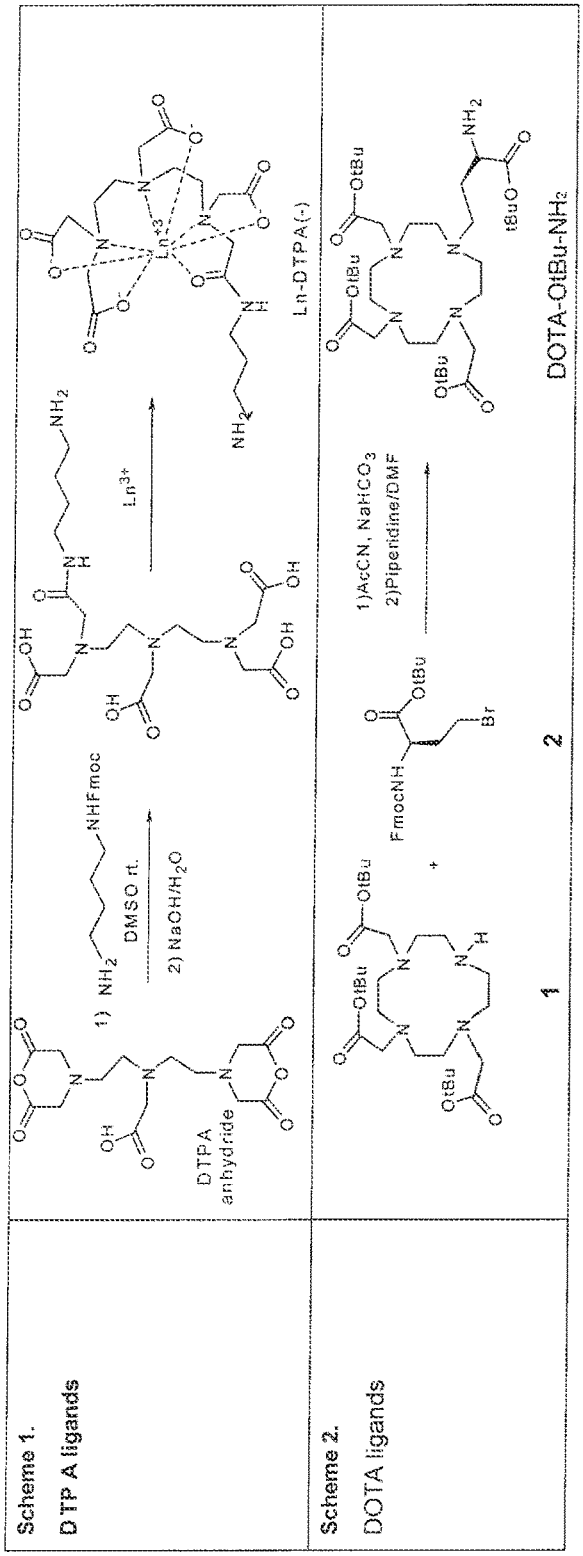

Scheme 1.
DTPA ligands
DTPA anhydride
1) NH2
NHFmoc
DMSO rt.
2) NaOH/H2O
Ln3+
Ln-DTPA(-)
Scheme 2.
DOTA ligands
1
FmocNH
Br
1)AcCN, NaHCO3
2)Piperidine/DMF
2
DOTA-OtBu-NH2

Figure 2

Figure 3

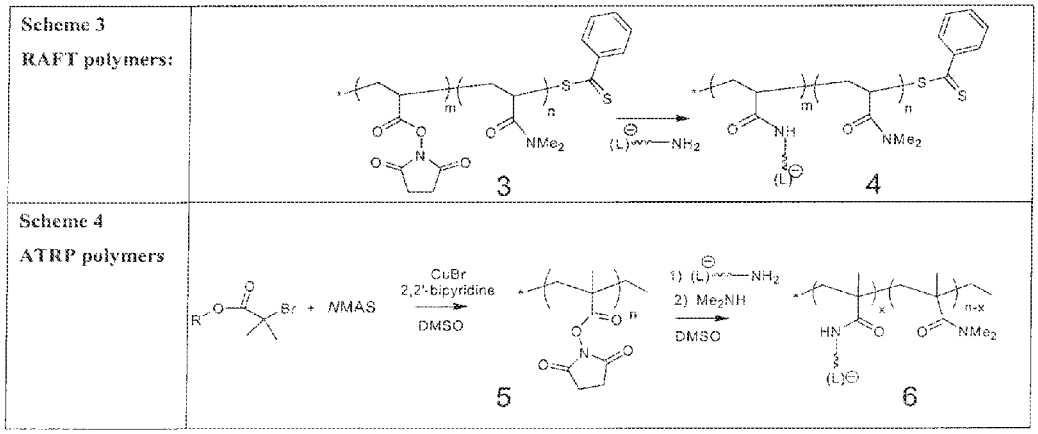

Scheme 3
RAFT polymers:
3
4
Scheme 4
ATRP polymers
NMAS
CuBr
2,2'-bipyridine
DMSO
5
1) (L)—NH2
2) Me2NH
DMSO
6

Figure 4

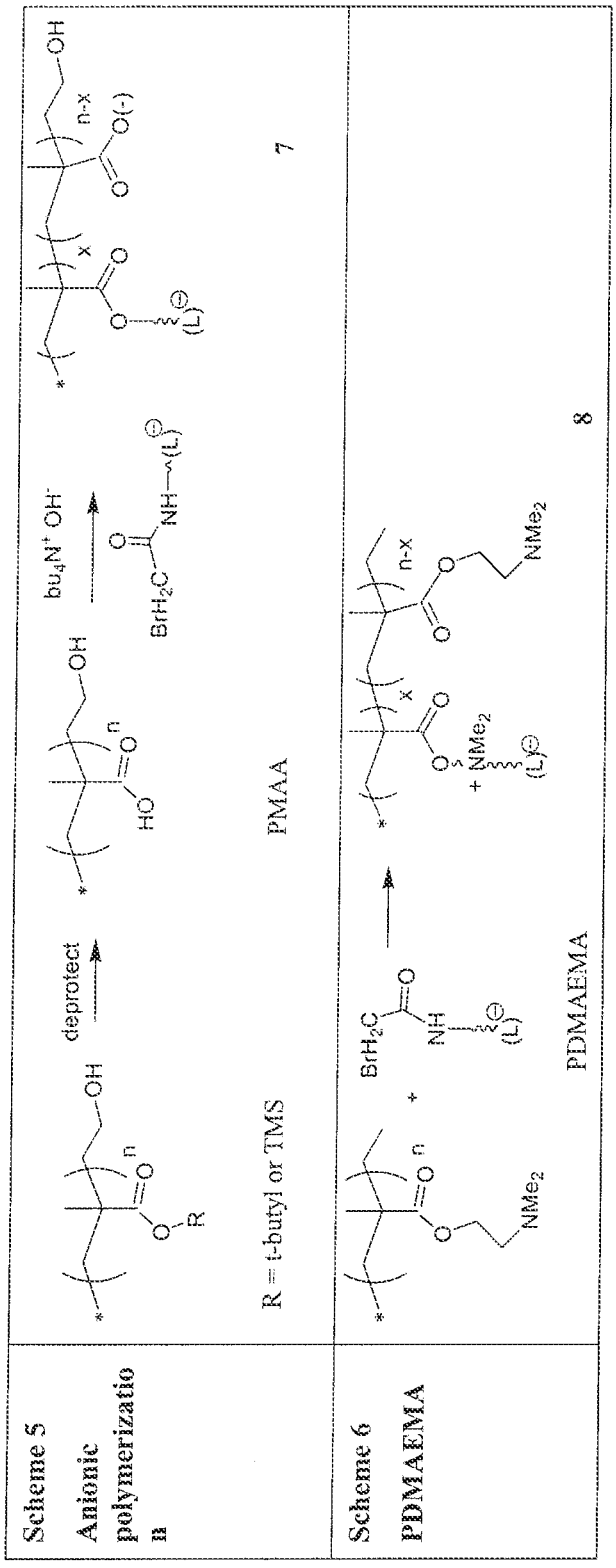

Scheme 5
Anionic polymerization
n
deprotect
R = t-butyl or TMS
PMAA
7
Scheme 6
PDMAEMA
PDMAEMA
8

Figure 5

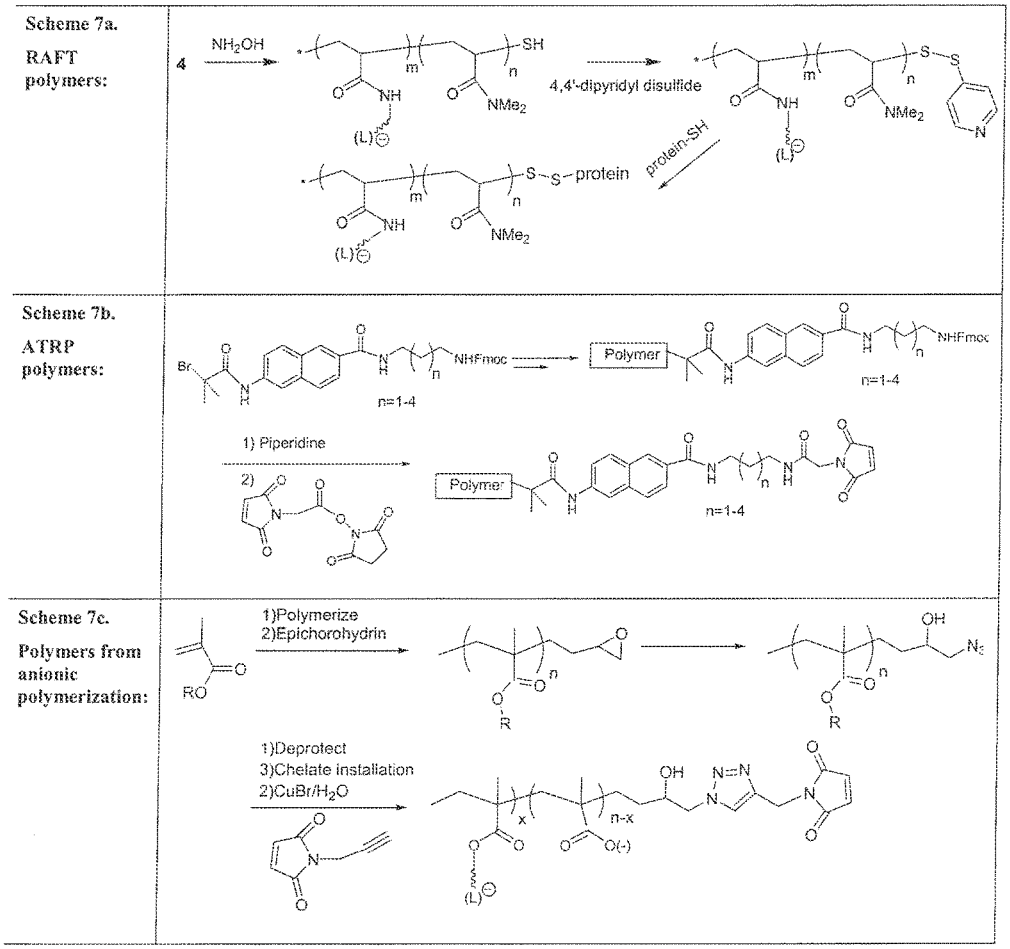
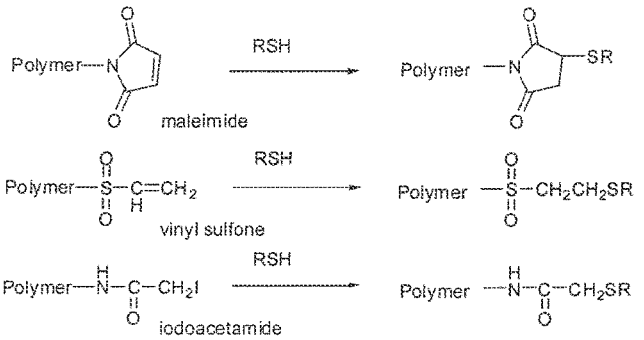

Scheme 7a.
RAFT polymers:
NH2OH
4,4'-dipyridyl disulfide
protein-SH
Scheme 7b.
ATRP polymers:
NHFmoc
Polymer
n=1-4
1) Piperidine
2)
Scheme 7c.
Polymers from anionic polymerization:
1)Polymerize
2)Epichorohydrin
1)Deprotect
3)Chelate installation
2)CuBr/H2O

Figure 6

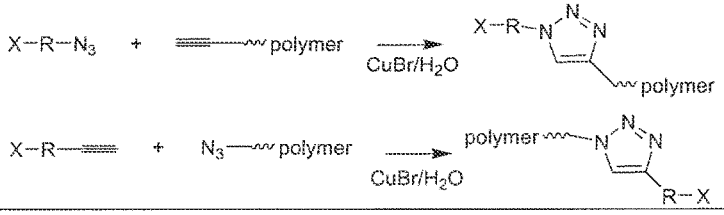
Scheme 8a.

Coupling through thiol coupling chemistry



Scheme 8b.

"click" chemistry for end-group attachment



Scheme 8c.

Coupling through the N-terminus or basic side chain (I) and through the C-terminus or acidic side chain (II).

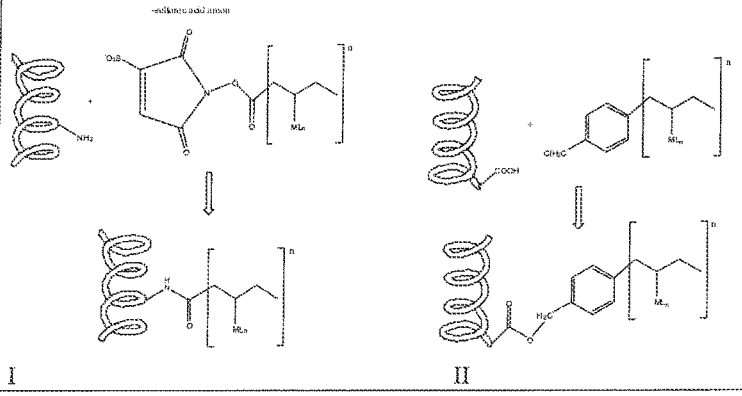
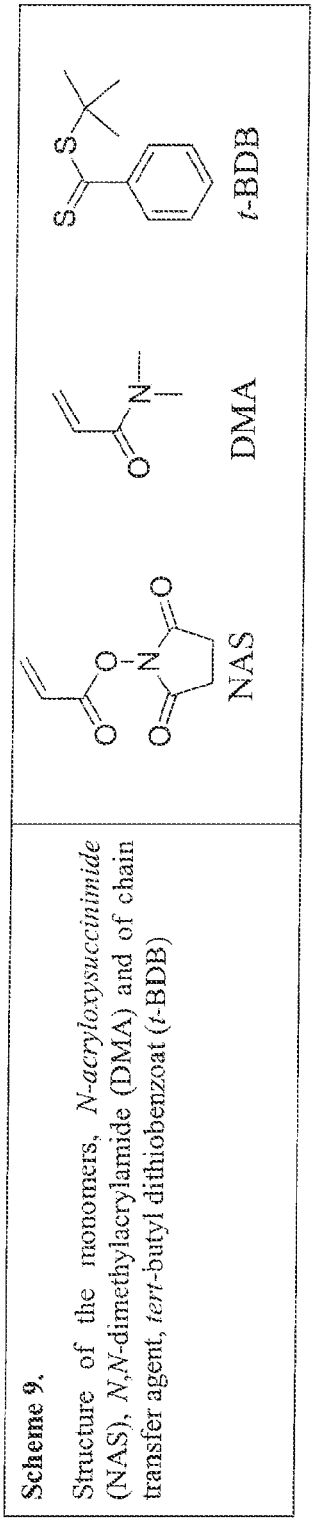


Figure 7



Scheme 9.

Structure of the monomers, *N-acryloxysuccinimide* (NAS), *N,N*-dimethylacrylamide (DMA) and of chain transfer agent, *tert*-butyl dithiobenzoat (*t*-BDB)

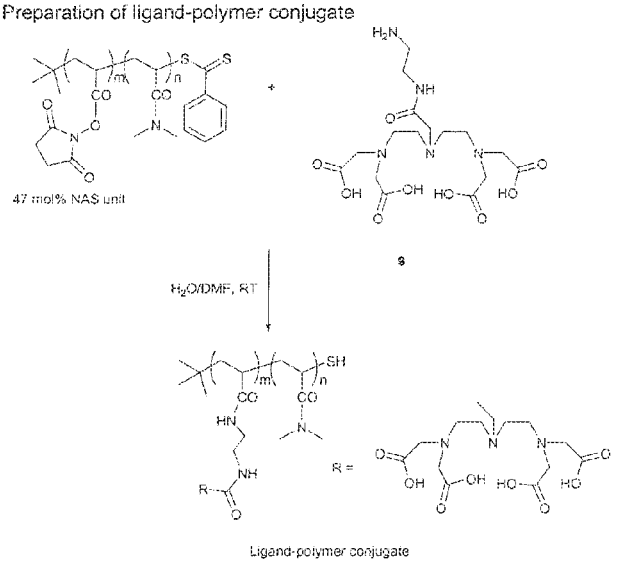
Figure 8

Ratio of NAS/DMA Mol%	Wt%	$[M]_0$ /$[CTA]_0$	$[CTA]_0$ /$[AMBN]_0$	Reaction time (h)	Polymer yield	$M_{n,GPC}$	M_w /M_n
13	20	70	1.4	18	75%	6500	1.45
47	60	55	1.5	18	80%	8298	1.50
60	72	70	3	18	80%	8000	1.15

CTA: Chain transfer agent, *t*-BDB. $[M]_0$: Starting monomer concentration. $M_{n,GPC}$: using polystyrene standards.

Figure 9

Scheme 10.
Preparation of ligand-polymer conjugate



Scheme 11.
Reaction of ligand-polymer conjugate with 1,4-bis(maleimido)butane

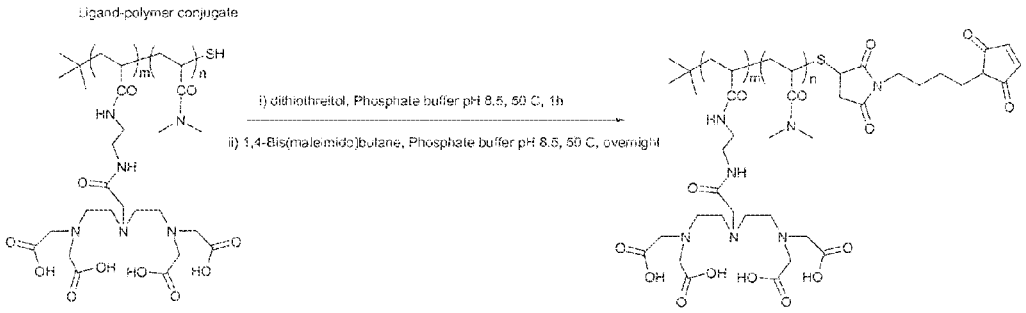
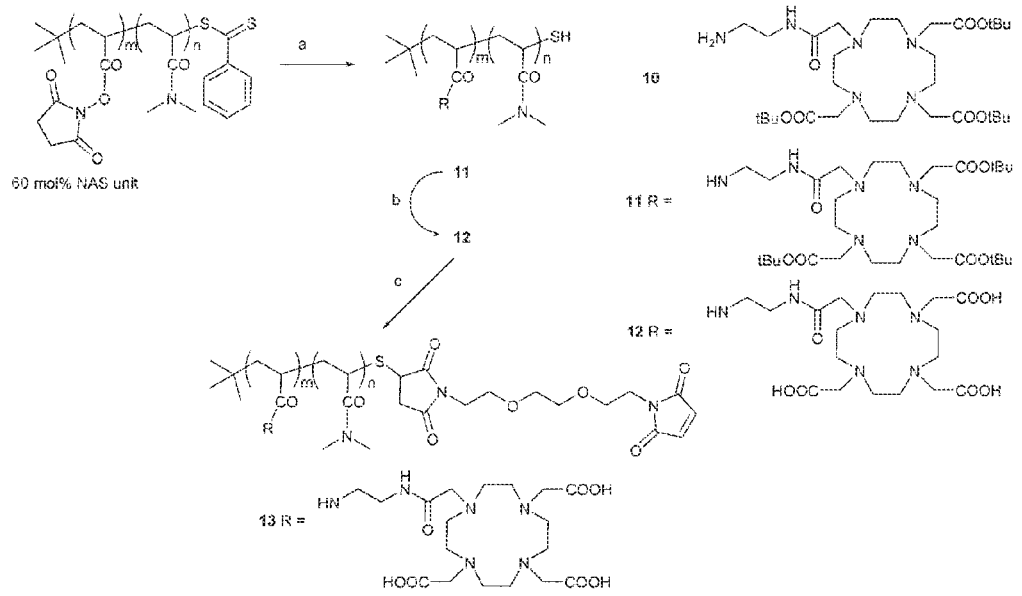


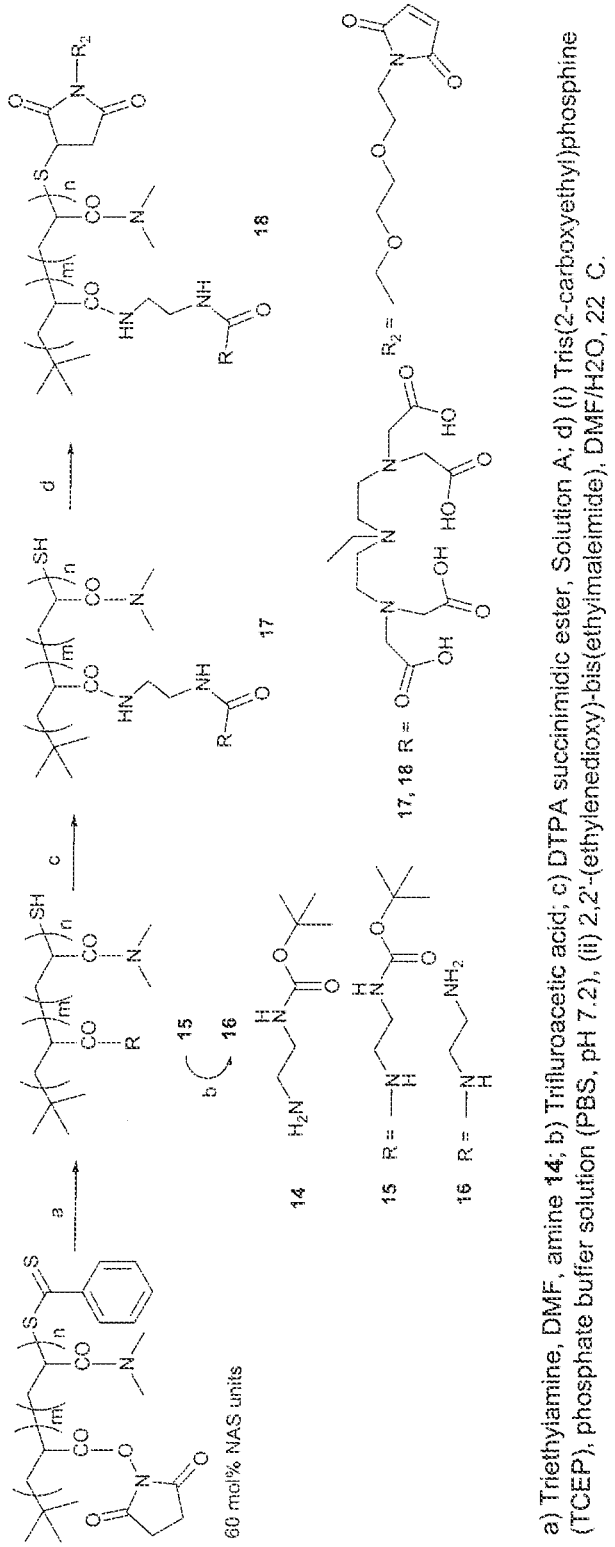
Figure 10

Scheme 12.
Preparation of DOTA based ligand-polymer conjugate



a) triethylamine, DMF, amine **10**, 14 h; b) Trifluoroacetic acid, 14 h;
c) (i) dithiothreitol, phosphate buffer pH 8.5, 50 C, 1 h,
(ii) 2,2'-(ethylenedioxy)bis(ethylmaleimide), DMF/H_2O, 1 h, RT

Figure 11



a) Triethylamine, DMF, amine **14**; b) Trifluroacetic acid; c) DTPA succinimidic ester, Solution A; d) (i) Tris(2-carboxyethyl)phosphine (TCEP), phosphate buffer solution (PBS, pH 7.2), (ii) 2,2'-(ethylenedioxy)-bis(ethylmaleimide), DMF/H2O, 22 C.

Figure 12

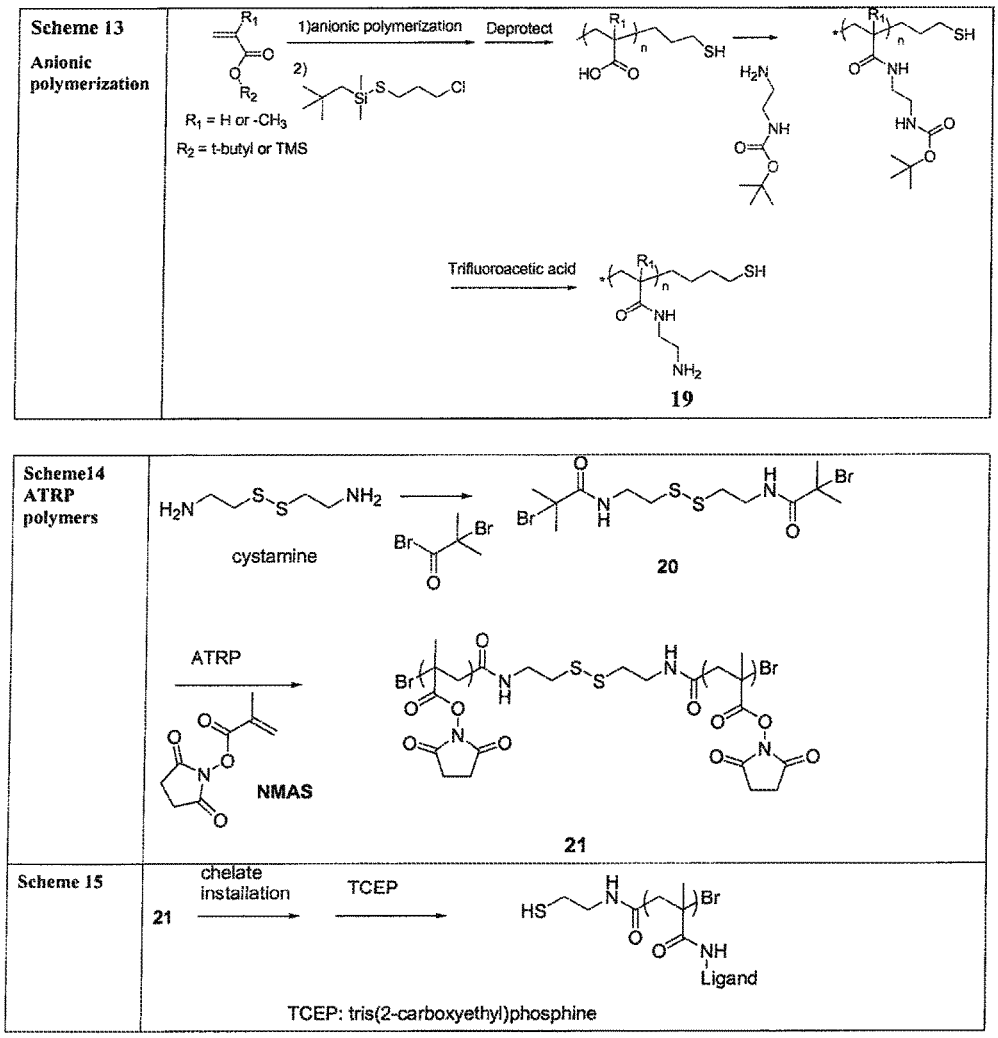

Scheme 13
Anionic polymerization
1)anionic polymerization
Deprotect
2)
R_1 = H or -CH_3
R_2 = t-butyl or TMS
Trifluoroacetic acid
19
Scheme14
ATRP
polymers
cystamine
20
ATRP
NMAS
21
Scheme 15
chelate installation
TCEP
21
Ligand
TCEP: tris(2-carboxyethyl)phosphine

Figure 13

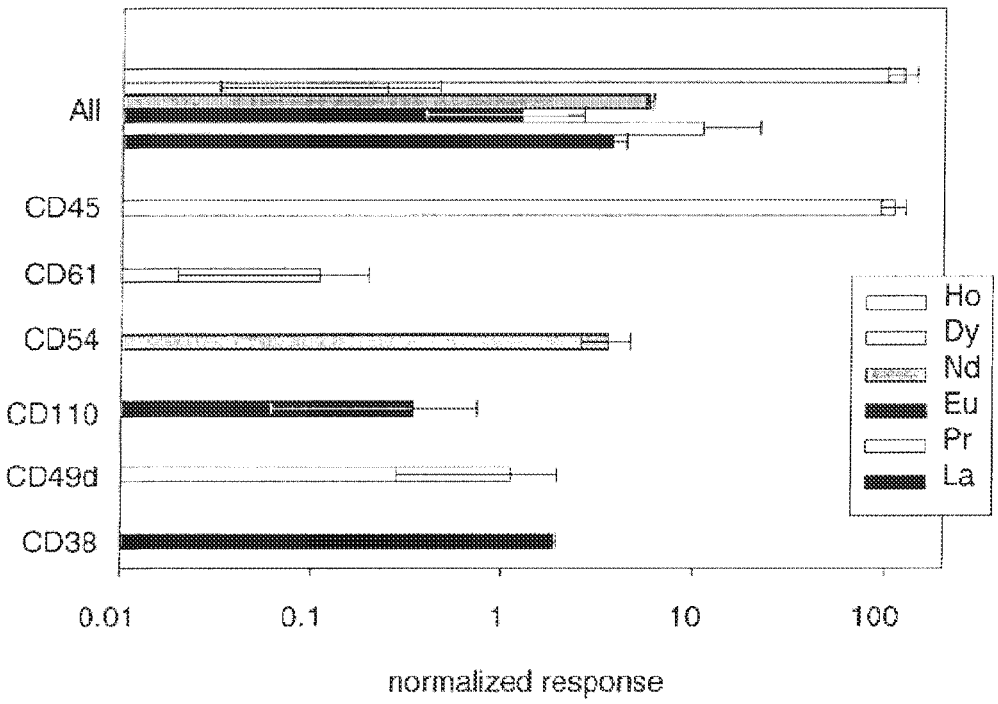
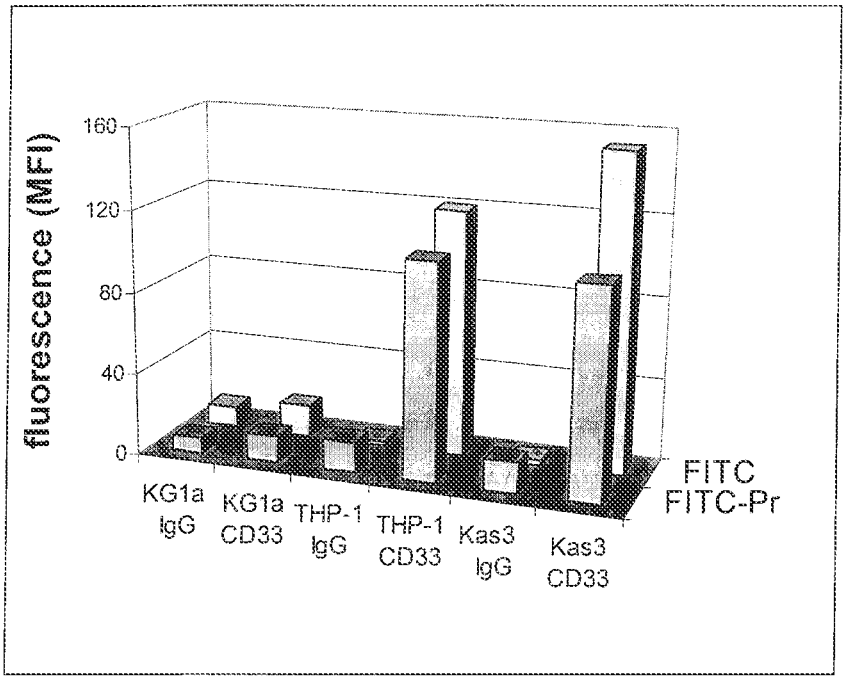
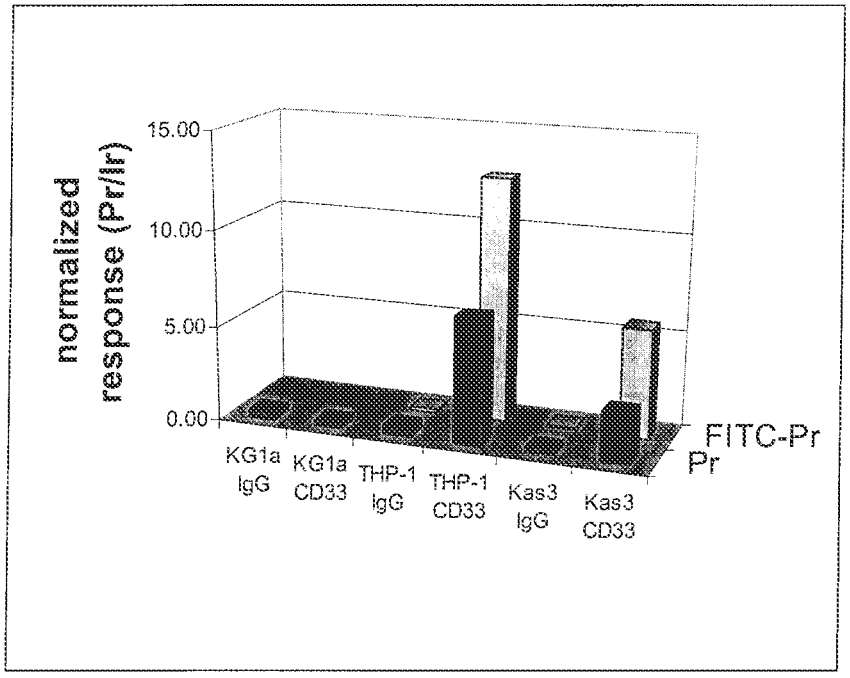

All
CD45
CD61
CD54
CD110
CD49d
CD38
Ho
Dy
Nd
Eu
Pr
La
0.01
0.1
1
10
100
normalized response

Figure 14


fluorescence (MFI)
160
120
80
40
0
KG1a IgG
KG1a CD33
THP-1 IgG
THP-1 CD33
Kas3 IgG
Kas3 CD33
FITC
FITC-Pr

a


normalized response (Pr/Ir)
15.00
10.00
5.00
0.00
KG1a IgG
KG1a CD33
THP-1 IgG
THP-1 CD33
Kas3 IgG
Kas3 CD33
FITC-Pr
Pr

b

Figure 15

Flow Chart I

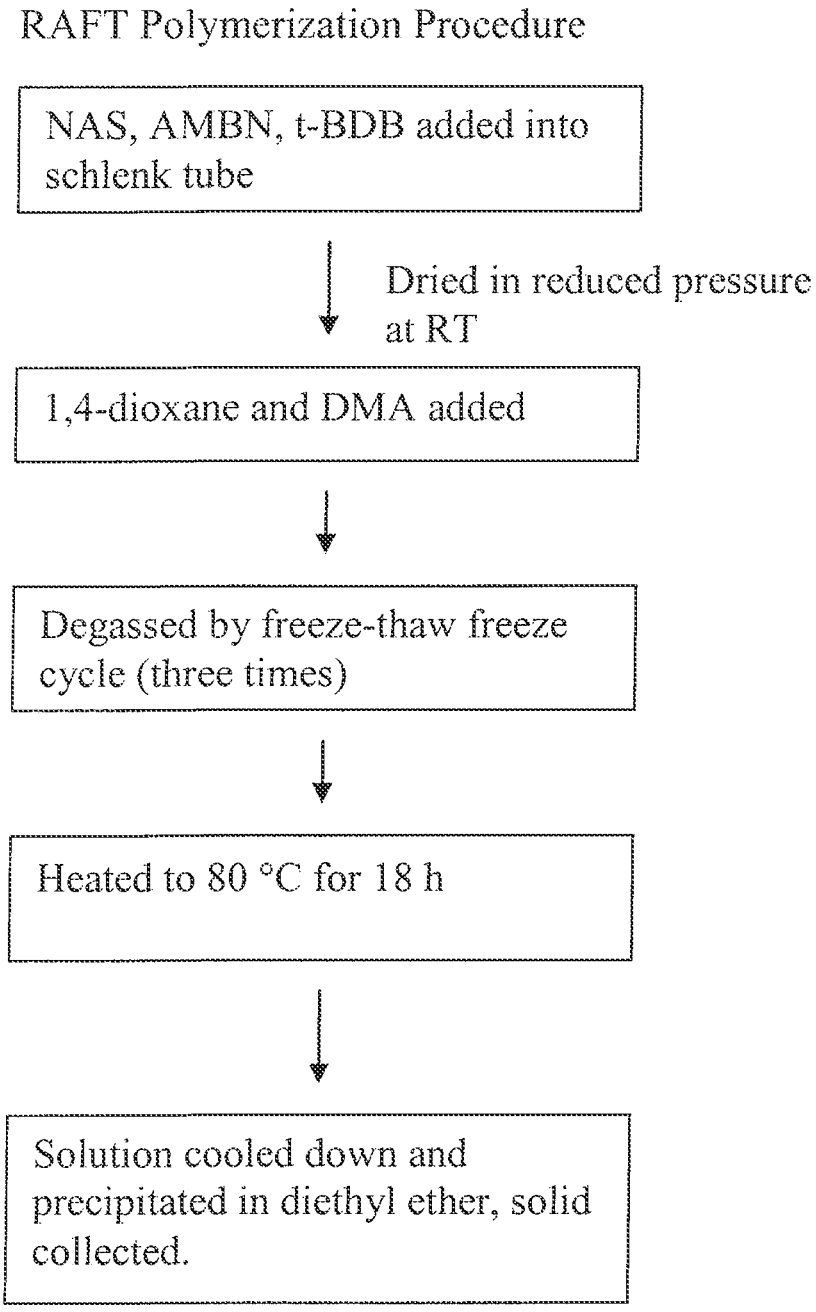
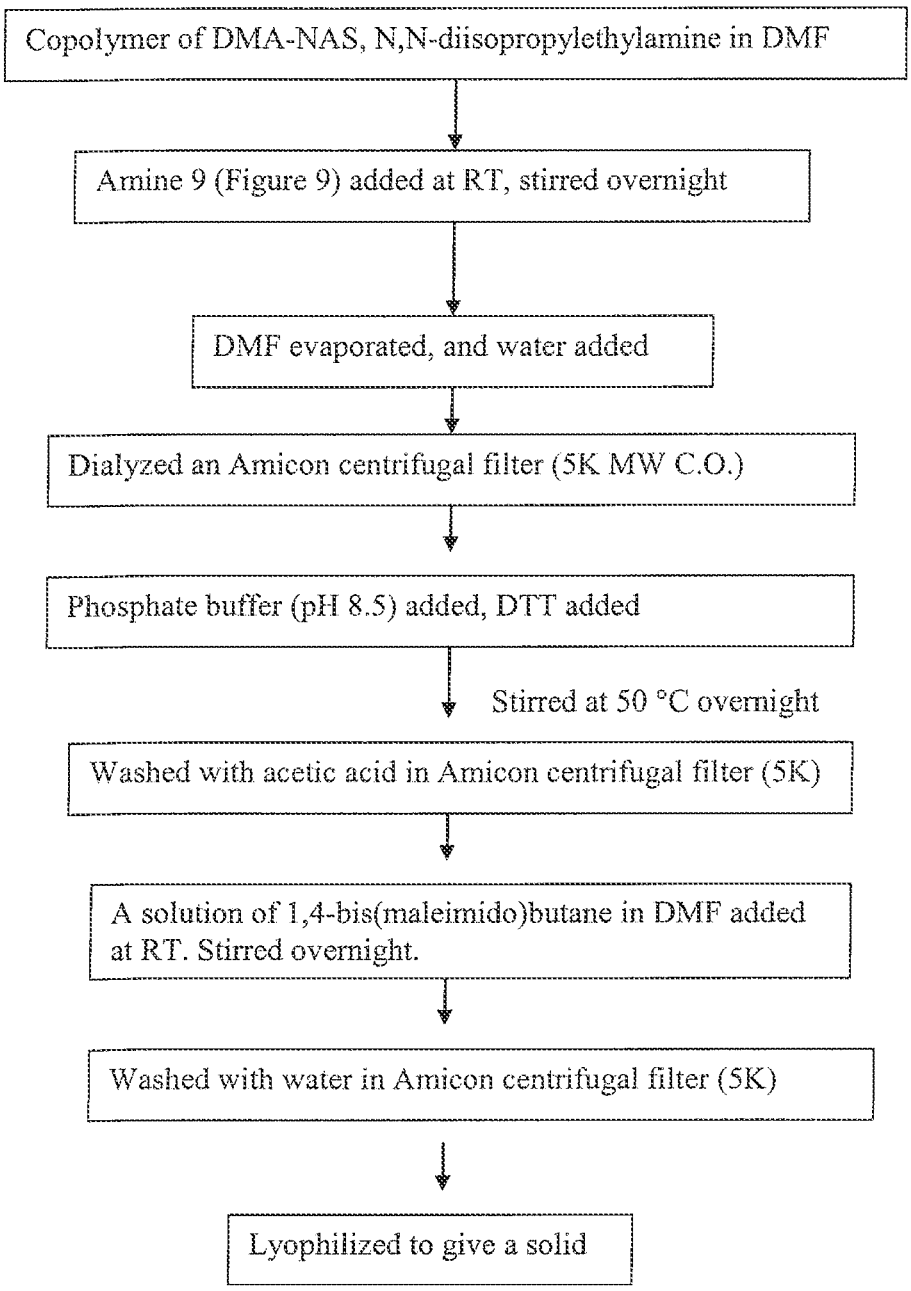


Figure 16
Flow Chart II

Polymer-DTPA-Linker attachment procedure (corresponding to Figure 9)



POLYMER BACKBONE ELEMENT TAGS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a divisional of U.S. application Ser. No. 14/659,224, filed on Mar. 16, 2015, now allowed, which is a divisional application of U.S. application Ser. No. 11/754,340 filed on May 28, 2007, now U.S. Pat. No. 9,012,239, which claims the benefit of U.S. Provisional Patent Application Ser. No. 60/803,356, entitled "Polymer backbone elemental tags," filed May 27, 2006, the entire contents of which are incorporated by this reference.

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FIELD

The invention relates to a new class of tagged biomolecules that have been specifically designed to operate in conjunction with elemental analysis detection, to provide high sensitivity multiplexed biomarker determinations.

INTRODUCTION

Technology that enables accurate protein quantitation is desired in the booming area of proteomics and drug discovery as well as in areas of clinical and diagnostic testing. It is also important in biological research aimed at analyzing protein synthesis, function and disease at the molecular level. Currently, there are several existing techniques that are widely used for estimating protein concentration including: Bradford and Lowry assays, UV spectroscopy, organic mass spectrometry, HPLC, flow cytometry, ligand binding assays, ELISA (Enzyme Linked Immunosorbent Assay), and RIA (RadioImmunoAssay). Nevertheless, this extensive assortment of well-established analytical tools and research techniques remains insufficient for today's challenges. The failures of these methods relate to limitations in sensitivity, selectivity, dynamic range, and the ability to determine the concentration of several proteins simultaneously in an accurate and absolute manner (multiplexing). The realization that elemental analysis offers significant advantages to the field of protein quantitation has directed the development of several new methods of protein quantitation via Inductively Coupled Plasma Mass Spectrometry (ICP-MS) linked immunoassays[1-4]. This new technique provides an innovative arena for ICP-MS in the analysis of biological samples[5; 6]. The unique analytical properties of ICP-MS allow the selection of tags from the non-radioactive elements that do not naturally occur in biological samples.

DEFINITIONS

"Elemental analysis" is a process where a sample is analyzed for its elemental composition and/or isotopic composition. Elemental analysis can be accomplished by a number of methods, including, but not limited to:

(i) optical atomic spectroscopy, such as flame atomic absorption, graphite furnace atomic absorption, and inductively coupled plasma atomic emission, which probe the outer electronic structure of atoms;

(ii) mass spectrometric atomic spectroscopy, such as inductively coupled mass spectrometry, which probes the mass of atoms;

(iii) X-ray fluorescence, particle induced x-ray emission, x-ray photoelectron spectroscopy, and Auger electron spectroscopy which probes the inner electronic structure of atoms.

"Elemental analyzer" is an instrument for the quantitation of the atomic composition of a sample employing one of the methods of elemental analysis.

"Particle elemental analysis" is a process where a sample, composed of particles dispersed in a liquid (beads in buffer, or cells in growth media, or blood, for example), is interrogated in such manner that the atomic composition is recorded for individual particles (bead-by-bead, cell-by-cell, particle-by-particle, for example). An example of the analytical instrument is a mass spectrometer-based flow cytometer, ICP-TOF, or ICP-MS or any elemental analyzer configured to interrogate individual particles.

"Volume or bulk elemental analysis" is a process where an analyzed sample is interrogated in such manner that the atomic composition is averaged over the entire volume of the sample.

"An internal standard" is defined as a known amount of a compound, different from the analyte that is added to the unknown. Signal from analyte is compared with signal from the internal standard to find out how much analyte is present. An internal standard may be used when performing mass spectrometry quantitation. An internal standard can be also used by other means known to those skilled in the art.

"Biological sample" refers to any sample of a biological nature that requires analysis. For example, the sample may comprise or may be suspected of comprising biological molecules, tissue, fluid, and cells of an animal, plant, fungus, or bacteria. It also includes molecules of viral origin. Typical samples include, but are not limited to, sputum, blood, blood cells (e.g., white cells), tissue or fine needle biopsy samples, urine, peritoneal fluid, and pleural fluid, or cells therefrom. Biological samples may also include sections of tissues such as frozen sections taken for histological purposes. Another typical source of biological samples are viruses and cell cultures of animal, plant, bacteria, fungi where gene expression states can be manipulated to explore the relationship among genes. Biological samples may also include solutions of biological molecules (either purified or not purified) such as proteins, peptides, antibodies, DNA, RNA, aptamers, polysaccharides, lipids, etc. Other examples are known to those skilled in the art.

"Antibodies" are immunoglobulin glycoprotein molecules found normally in serum of animals. Antibodies may be made in mammals such as rabbits, mice, rats, goats, etc., and chicken or may be made by recombinant methods as is known to those skilled in the art and described, for example, in U.S. Pat. No. 4,816,567. Procedures for immunization and elicitation of a high antibody production response in an animal are well known to those skilled in the art and can be found, for example, in Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, 1988, pages 92-115. Antibodies may be used as whole molecules, fragments of molecules known as Fab' and Fab2' fragments, as monovalent antibodies (combining a light chain and a modified heavy chain), and other examples known in to those skilled in the art.

"Primary antibodies" are antibodies that bind specifically to an antigen injected into an animal. They can be extracted from the animal or can be made by recombinant means.

"Secondary antibodies" are those antibodies that bind specifically to primary antibodies. For example, primary antibodies can be used as the antigen injected into an animal of a different species, to generate secondary antibodies. For example, rabbit secondary anti-mouse antibodies can be made by immunizing a rabbit with mouse antibodies.

"Antigen" is a substance that stimulates an immune response in a host organism, especially the production of antibodies. Antigens are usually proteins or polysaccharides, but can be any type of molecule, including but not limited to, small molecules (haptens) coupled to a carrier-protein.

"Bio-markers" are molecules and constructs, which may for example be antigens, small molecules, nucleotides, oligonucleotides, DNA or RNA, that are present in the cell volume or on the cell surface of only one type of cell, or whose relative abundance is unique to that type of cell. Cell bio-markers can be used to distinguish that cell from other cells. For example, antigens present on the cell surface of only one type of cell are called cell surface bio-markers that distinguish that cell from other cells.

"Immunoassay" as used herein means an assay in which an analyte, such as cellular antigen or bio-marker, is detected by an affinity reagent such as a primary antibody. For example, an "immunoassay" can be an assay in which an analyte is detected by a tagged affinity reagent, such as a primary antibody conjugated to a metal tagged polymer.

"Biomolecule" as used herein means any biological molecule and includes small biomolecules, for example, but not limited to: Lipids, Phospholipids, Glycolipids, Sterols, Vitamins, Hormones, Neurotransmitters, Carbohydrates, Sugars, Disaccharides, Amino acids, Nucleotides, Phosphate, and Monosaccharides; and large biomolecules, for example but not limited to: Peptides, Oligopeptides, Polypeptides, Proteins, Nucleic acids, i.e. DNA, RNA, Oligosaccharides, Polysaccharides, and Prions. Other biomolecules are known to those skilled in the art and are encompassed in the applicant's teachings.

"Affinity reagent" is a biomolecule capable of tightly binding to a target molecule or analyte, for example an antigen or biomarker. For example, an antibody is an affinity reagent that recognizes and binds with high affinity to a specific antigen. Streptavidin is a protein molecule that specifically binds biotin and may be considered as another example of the affinity reagent. Other affinity reagents are known to those skilled in the art, and include, but are not limited to aptamers, oligonucleotides, protein molecules, lectins and polysaccharides.

"Tagged affinity reagent" is an affinity reagent (for example, an antibody or aptamer or oligonucleotide, polysaccharides, lipids, hormones, growth factors) that is conjugated to a synthetic tag (moiety) usually through a linker group. The tag can be, but is not limited to, a polymer with covalently attached multiple chelating groups. To a greater extent, the chelating groups can have an element or multitude of elements attached to them. The sequence and order of the chelation stage depends on the tagging protocol.

The term "detect" is used in the broadest sense meaning to include both qualitative and quantitative measurements of a specific molecule. For example, qualitative and quantitative measurements of a specific antigen or biomarker with the help of a tag (for example, a tagged antibody or other tagged affinity reagent).

"Element tag" or "tag" is a chemical moiety which includes an element or multitude of elements having one or many isotopes (referred to as "tag atoms") attached to a supporting molecular structure, or that is capable of binding said element(s) or isotope(s). The element tag can also comprise the means of attaching the element tag to a molecule of interest or target molecule (for example, an analyte). Different element tags may be distinguished on the basis of the elemental composition of the tags. An element tag can contain many copies of a given isotope and can have a reproducible copy number of each isotope in each tag. An element tag is functionally distinguishable from a multitude of other element tags in the same sample because its elemental or isotopic composition is different from that of the other tags.

The term "tag atom" is the atom of the element or isotope that differentiates one element tag from another and that is detected by elemental analysis.

"A support" is a surface which has been functionalized by, for example, pyrrole-2,5-dione (maleimido), sulfonic acid anion, or p-(chloromethyl) styrene. A support, for example, may be but is not limited to, a synthetic membrane, bead (polystyrene, agarose, silica, etc), planar surface in plastic microwells, glass slides, reaction tubes, etc. as is known to those skilled in the art.

"ICP-MS" is the Inductively Coupled Plasma Mass Spectrometer—a sensitive mass spectrometry based elemental analyzer. Different ICP-MS configurations are primarily distinguished by the mass selecting technique employed and can be, for example the quadrupole or time-of-flight (ICP-TOF) or magnetic sector (high resolution ICP-MS). There are many commercially available ICP-MS models having a wide spectrum of configurations, capabilities and modifications.

A "polymer" is a substance composed of molecules characterized by the multiple repetitions of one or more species of atoms or groups of atoms (constitutional units) linked to each other in amounts sufficient to provide a set of properties that do not vary markedly with the addition or removal of one or a few constitutional units. (IUPAC definition, see E. S. White, J. Chem. Inf. Comput. Sci. 1997, 37, 171-192). A polymer molecule can be thought of in terms of its backbone, the connected link of atoms that span the length of the molecule, and the pendant groups, attached to the backbone portion of each constituent unit. The pendant groups are often chemically and functionally different from the backbone chain. Pendant groups that have a high affinity for metal ions can act as chelating groups or ligands for those ions.

"Copolymers" are polymers that consist of two or more chemically different constituent units. A "linear polymer" is a polymer characterized by a linear sequence of constituent units. A "block copolymer" is a linear polymer with sequences of constituent units of a common type, joined to sequences of constituent units of a different type. A "branched polymer" is a polymer in which additional polymer chains (the branches) issue from the backbone of the polymer. One commonly refers to the longest linear sequence as the "main chain". A branched polymer in which the chemical composition of the constituent units of the branch chains is different than those of the main chain is called a "graft copolymer".

"Star polymers" have multiple linear polymer chains emanating from a common constituent unit or core. "Hyperbranched polymers" are multiple branched polymers in which the backbone atoms are arranged in the shape of a tree. These polymers are related to "dendrimers", which have three distinguishing architectural features: an initiator core, interior layers (generations) composed of repeating

units radially attached to the initiator core, and an exterior surface of terminal functionality attached to the outermost generation. "Dendrimers" differ from hyperbranched polymers by their extraordinary symmetry, high branching, and maximized (telechelic) terminal functionality.

A "metal tagged polymer" (also a "polymeric metal tag carrier", or "metal-polymer conjugate", or "chelate-derivatized polymer") is a variety of the element tag which consists of a polymer backbone bearing at least one pendant chelating group with metal atoms attached to them. These metal tagged polymers can be, but are not limited to, linear, star, branched, or hyperbranched homopolymers or copolymers as well as block or graft copolymers.

A "metal binding pendant group" is a pendant group on the polymer that is capable of binding a metal or an isotope of a metal. It can also be referred to as a ligand.

An "attachment (linker) group" or "linker" is a portion of a molecule that is used to couple (conjugate) two different molecules or polymers, two subunits of a molecule, or a molecule to a substrate, for example an affinity agent.

Commonly used abbreviations: NAS is N-acryloxysuccinimide; NMAS is N-methacryloxysuccinimide; DMA is N,N-dimethylacrylamide; t-BDB is the reversible addition-fragmentation chain transfer (RAFT) chain transfer agent, tert-butyl dithiobenzoate; AMBN is 2,2-azobis(2-methylbutyronitrile); DMSO is Dimethyl Sulfoxide; DOTA is 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; PMAA is poly(methacrylic acid); DTPA is diethylenetriamine pentaacetic acid; PDMAEMA is poly(dimethylaminoethyl methacrylate); Fmoc is 9-fluorenylmethyl carbamate; DTT is dithiothreitol; TMS is trimethylsilyl and TCEP is tri(2-carboxyethyl)phosphine.

The terms Mn, Mw and PDI (polydispersity index): Mw/Mn are used to indicate the number and average molecular weight and the polydispersity index describes the molecular weight distribution, respectively.

"Chelation" is the process of binding of a ligand, the chelant, chelator or chelating agent, to a metal ion, forming a metal complex, the chelate. In contrast to the simple monodentate ligands like H_2O or NH_3, the polydentate chelators form multiple bonds with the metal ion.

"Transition element" means an element having one of the following atomic numbers 21-30, 39-48, 57-80 and 89-92. Transition elements include the rare earth metals, lanthanides and noble metals.

"Lanthanides" are the transition metals with atomic numbers from 57 to 71 including La, Ce, Pr, Nd, Pm, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu.

"Metal" means an element having one of the following atomic numbers 3, 4, 11-13, 19-33, 37-52, 55-84, 87-102.

SUMMARY

A class of tags optimized for elemental analysis including (but limited to) the ICP-MS application has not before been developed. Preliminary studies had to be done[1;3;4;7] using tags that are currently in use for completely different purposes. The element tags of the present invention are not those of the prior art and are specifically designed for elemental analysis. To implement elemental tagging to its fullest, the development of a new class of tags was required. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) has a number of unique properties that can be harnessed to create an ideal elemental tag instrument combination. The most important advantage is the fact that a large number of heavy metals and their isotopes provide distinct signals that can be detected simultaneously. Thus many, for example greater than 50, element tags can be developed; the obtained intensity of tag elements serves as a signature of the analyte concentration in the sample. Secondly, the abundance sensitivity of ICP-MS, a measure of the overlap of signals of neighboring isotopes, is large (for example greater than 10^6 for the quadrupole analyzer), and this ensures independence of the detection channels over a wide dynamic range. The third key property is that MS is very sensitive; detection on the order of 100 molecules of a given antigen per cell may be feasible, and largely independent of the order of multiplex, a substantial improvement over current fluorescence cytometer instruments. Finally, ICP-MS as a detector offers absolute quantification that is largely independent of the analyte molecular form or sample matrix. There is a definite need to integrate these key properties of elemental analysis with bio-analytical methodology. Here, we provide a novel design of the element tags, which ensures dramatically higher multiplex capability and sensitivity of bio-assays.

The new class of polymer based element tags is suitable for determination using conventional ICP-MS instruments in the instance that an average assay over a sample ensemble (i.e., bulk assay) is desired. For example, where a tissue is sufficiently homogeneous, or the diagnostic allows for averaging over the biopsy, the sample may be stained with the metal-tagged affinity reagents and, following washing, may be acidified to lyse the cells of the tissue and provide a homogeneous solution that can be analyzed according to long-standing standard ICP-MS protocols. The bulk assay protocol still allows for massively multiplexed assay, with detection limits for each marker comparable to individual radio-immunoassay. Cell biologists might view this as a quantitative highthroughput analog of Western blotting.

The new class of polymer based element tags is suitable for determination using a novel flowcytometry ICP-MS based instrument[8] and provide up to 50 or more distinguishable reporter tags for immunological assays that enable the simultaneous determination (massively multiplexed) of many biomarkers, ultimately providing exquisite distinction and identification of diseased cells (or other cells of interest) in patient's samples in particle elemental analysis.

The new class of polymer based element tags is suitable for double labeling of affinity reagents—fluorescent label and element tag on the same affinity reagent. Previously, double labeled antibodies were used to localize specific cell types in tissue sections (fluorescent microscopy) and then identify the particular structures of cells using electron microscopy. Therefore, antibodies were labeled with fluoresceneisothiocyonate (FITC) and ferritin as an electron dense material.[9] More recently, immunoprobes that combine a fluorescent label with a small gold cluster have been prepared by covalent conjugation with Fab' fragments. These new immunoconjugates allow the collection of two complementary sets of data, from fluorescence and electron microscopy, from a single labeling experiment.[10] Another advance in reagents such as terbium-fluorescein and terbium-green fluorescent protein fluorescence resonance energy transfer pairs was achieved to study kinase reactions using Time Resolved-Fluorescence Resonance Energy Transfer (TR-FRET)[11].

The Applicant's teaching includes double labeled affinity reagents to facilitate presorting and subsequent elemental analysis of rare cells in mixed samples by ICP-MS-based flow cytometry. Cell biology requires microscopic localization of biomarkers on the cell surface or intracellularly. At the same time, quantitative information on the abundance of the markers is necessary. By covalently attaching a fluorescent label and an element tag to the same affinity reagent (for

example an antibody) and using this affinity reagent, first, to localize the signal to a particular subcellular structure (membrane, nucleus, cytoplasm, cytoskeleton, etc) via fluorescent microscopy and, second, to quantify the number of bound affinity reagents by ICP-MS, will significantly increase biological understanding of processes under investigation.

An aspect of the invention is to provide an element tag comprising a polymer, wherein the polymer comprises at least one metal-binding pendant group that comprises at least one metal atom or is capable of binding at least one metal atom. The element tag can further comprise a functional group that allows the polymer to be attached to one of a linker, a spacer, or a biomolecule. The element tag can be water soluble. It can also be negatively charged. The number of metal-binding pendant groups capable of binding at least one metal atom can be between approximately 1 and 1000, and most typically between approximately 10 and 250. At least one metal atom can be bound to at least one of the metal-binding pendant groups. The polymer can have a degree of polymerization of between approximately 1 and 1000, and most typically between 10 and 250.

The polymer can be selected from the group consisting of linear polymers, copolymers, branched polymers, graft copolymers, block polymers, star polymers, and hyperbranched polymers. The backbone of the polymer can be derived from substituted polyacrylamide, polymethacrylate, or polymethacrylamide and can be a substituted derivative of a homopolymer or copolymer of acrylamides, methacrylamides, acrylate esters, methacrylate esters, acrylic acid or methacrylic acid.

The metal-binding pendant group can be attached to the polymer through an ester or through an amide. The functional group can be a thiol-reactive group. The metal atom can be a transition element or an isotope thereof, or a lanthanide or an isotope of a lanthanide. The element tag can further comprise a linker attached to the functional group of the polymer, wherein the linker is capable of covalent attachment to a biomolecule. The element tag can further comprise a spacer attached to the linker, wherein the spacer is capable of attachment to a biomolecule. The spacer can be a polyethylene glycol (PEG) spacer. The spacer can comprise a functional group that is capable of binding the spacer to the polymer via a spacer-reactive functional group on the polymer. Further the spacer can contain a functional group that is capable of binding a linker to the spacer.

The element tag described above, can be covalently attached to a biomolecule. The biomolecule can be an affinity reagent, and the affinity reagent can be an antibody.

Another aspect of the invention is to provide an element tagged affinity reagent, wherein the affinity reagent is tagged with the element tag described above, and wherein at least one of the pendant groups binds, or is capable of binding, at least one metal atom.

Another aspect of the invention is to provide a method of preparing the element tag described above, comprising: (i) providing a polymer; and (ii) covalently attaching at least one metal-binding pendant group containing at least one metal atom or capable of binding at least one metal atom to the polymer. The step of providing the polymer can comprise synthesis of the polymer wherein the synthesis is selected from the group consisting of reversible addition fragmentation polymerization (RAFT), atom transfer radical polymerization (ATRP) and anionic polymerization. The step of providing the polymer can comprise synthesis of the polymer from compounds selected from the group consisting of N-alkyl acrylamides, N,N-dialkyl acrylamides, N-aryl acrylamides, N-alkyl methacrylamides, N,N-dialkyl methacrylamides, N-aryl methacrylamides, methacrylate esters, acrylate esters and functional equivalents thereof. The metal-binding pendant group that is capable of binding at least one metal atom can comprise a diethylenetriaminepentaacetate (DTPA) ligand or a 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) ligand. The method can further comprise functionalizing the polymer, wherein the functional group is capable of covalently binding a biomolecule. The method can further comprise attaching a linker to the functional group of the polymer, wherein the linker is capable of binding a biomolecule. The method can further comprise covalently binding a biomolecule to the linker. Finally, the method can further comprise binding at least one metal atom to at least one metal-binding pendant group.

Another aspect of the invention is to provide a method of preparing the element tag described above, comprising: (i) providing a polymer comprising at least one metal-binding pendant group that contains at least one metal atom or is capable of binding at least one metal atom, and comprising a functional group that allows the polymer to covalently bind a linker; (ii) attaching a linker to the functional group of the polymer, wherein the linker is capable of binding a biomolecule; (iii) covalently binding a biomolecule to the linker; and (iv) binding at least one metal atom to at least one metal-binding pendant group. The step of binding at least one metal atom to at least one metal-binding pendant group can be performed before step (ii). The step of binding at least one metal atom to at least one metal-binding pendant group can be performed before step (iii). The step of binding at least one metal atom to at least one metal-binding pendant group can be performed after step (iii). The method can further comprise a step of attaching a spacer to the linker, wherein the spacer lies between the linker and the biomolecule and/or a step of attaching a spacer to the polymer, wherein the spacer lies between the polymer and the linker. The spacer can be added before step (ii). The spacer can be a polyethylene glycol (PEG) spacer. The spacer can contain a functional group that is capable of binding the spacer to the polymer via a spacer-reactive functional group on the polymer. The spacer can contain a functional group that is capable of binding the spacer to the linker. The method can include a step of reacting the thiol with a maleimido attachment group.

Another aspect of the invention is to provide an element tag prepared by the methods described above.

Another aspect of the invention is a method for the analysis of an analyte, comprising (i) incubating the element tagged affinity reagent described above with an analyte, wherein the affinity reagent binds with the analyte; (ii) separating unbound tagged affinity reagent from bound affinity reagent; and (iii) analyzing the element bound to the affinity reagent attached to the analyte by elemental analysis.

Another aspect of the invention is to provide a method for the multiplex analysis of two or more analytes, comprising: (i) incubating two or more differential element tagged affinity reagents described above with two or more analytes, wherein the affinity reagents bind with the analytes, to produce two or more differentially tagged analytes; (ii) separating unbound affinity reagents from bound affinity reagents; and (iii) analyzing the differential tags bound to the two or more analytes by elemental analysis.

Another aspect of the invention is to provide a method for the analysis of an analyte, comprising: (i) incubating the element tag described above with an analyte, so that the element tag binds the analyte; (ii) separating unbound tag

elements from bound tag elements; and (iii) analyzing the bound tag elements by elemental analysis.

The affinity reagent of any of the above methods can further labeled with a fluorescent label. The analyte can be located within or on a cell, for example a diseased cell, and further a leukemia cell. The step of analysis can comprise bulk analysis, wherein the atomic composition is averaged over an entire volume of a sample, and/or analysis of single particles. The particles can be cells.

The methods described above can be done by elemental analysis by ICP-MS or by a mass spectrometer based flow cytometer.

Another aspect of the invention is to provide a kit for the preparation of the element tag described above, comprising at least one of the following: a polymer comprising at least one metal-binding pendant group which comprises at least one metal atom or is capable of binding at least one metal atom and further comprising a functional group that allows the polymer to be attached to one of a linker, a spacer, or a biomolecule, a metal solution, reagents for the attachment of the linker, spacer or biomolecule to the polymer, reagents for attachment of a functional group to the linker or the spacer, reagents for attachment of a metal to the polymer, affinity reagents including antibodies, buffers, instructions for preparing the element tag, instructions for attaching the element tag to an affinity reagent, and instructions for attaching a metal to the element tag.

Another aspect of the invention is to provide a kit for the analysis of analytes according to the methods described above comprising at least one of the following: a polymer comprising at least one metal-binding pendant group which contains at least one metal atom or is capable of binding at least one metal atom and further comprising a functional group that allows the polymer to be attached to one of a linker, a spacer, or a biomolecule, a metal solution, reagents for the attachment of the linker, spacer or biomolecule to the polymer, reagents for attachment of a functional group to the linker or the spacer, reagents for attachment of a metal to the polymer, affinity reagents including antibodies, buffers, instructions for preparing the element tag, instructions for attaching the element tag to an affinity reagent, instructions for attaching a metal to the element tag. and instructions for using the element tags for the analysis of analytes by elemental analysis.

The polymer for any of the above kits can be selected from the group consisting of homopolymers or copolymers of acrylamides, methacrylamides, acrylate esters, methacrylate esters, acrylic acid and methacrylic acid. The reagents can include at least one of the following: TCEP (tri(2-carboxyethyl)phosphine), Ligand-Polymer-Linker-Spacer Conjugate, phosphate buffer, TBS (tris-buffered saline), EDTA (Diaminoethanetetraacetic acid), ammonium acetate buffer, antibodies, metal salt solution, lanthanide salt solution, blocker buffers, washing buffers, FBS (fetal bovine serum), DMEM (Dulbecco's Modified Eagle's Medium), BSA (bovine serum albumin), dithiothreitol, bismaleimide, and DMF (dimethylformamide). The polymer can be attached to a linker or it can be attached to a linker and a spacer.

These and other features of the applicant's teachings are set forth herein.

BRIEF DESCRIPTION OF THE FIGURES

The invention is illustrated in the figures of the accompanying drawings, which are meant to be exemplary and not limiting, and in which like references are intended to refer to like or corresponding parts.

FIG. **1**. Schematic views of the element tags for the detection of biomolecules which according to the invention have the general structure I. Proposed polymeric metal chelates: R=organic group, L=Metal ligand. In structure "a" each repeat unit of the polymer bears the liganded Ln3+, denoted by (L). In structure "b", a fraction of the repeat units have an organic group R according to the invention. Asterisk (*) represents the initiated end of the polymer NAS is schematic view of N-acryloxysuccinimide. NMAS is schematic view of N-methacryloxysuccinimide.

FIG. **2**. Schematic views of an example of the synthesis of functional ligands that can be used to attach the element/metal "L" to the polymer.

FIG. **3**. Schematic views of attaching ligands (pending groups) to the RAFT polymers (Scheme 3) and ATRP polymers (Scheme 4).

FIG. **4**. Schematic views of attaching ligands (pending groups) to polymers produced by anionic polymerization (Scheme 5) and PDMAEMA (Scheme 6).

FIG. **5**. Schematic views of attachment of the coupling group (the linker) to the RAFT polymers (Scheme 7a), ATRP polymers (Scheme 7b), and polymers produced by anionic polymerization (Scheme 7c).

FIG. **6**. Schematic views of alternative examples of coupling chemistry according to the invention. In scheme 8b, the term "end-group" is used to refer to the coupling group.

FIG. **7**. Schematic views of structures of monomers.

FIG. **8**. Experimental conditions and molecular weight data for random copolymers of DMA and NAS in dioxane at 80° C.

FIG. **9**. Schematic views of preparation of ligand-polymer conjugate.

FIG. **10**. Schematic views of preparation of the DOTA based ligand-polymer conjugate.

FIG. **11**. Schematic views of synthesis of the element tag.

FIG. **12**. Schematic views a process to generate a polymer with pendent amino groups for attachment of DTPA ligands and of employing a new initiator based on cystamine.

FIG. **13**. Is a bar graph of the results of Experiment 6. Growing K562 cells (non-differentiated) were stained with primary antibodies labeled with Ligand-Polymer Conjugate (as described in Scheme 11)—carrying identifying lanthanides: anti-CD38 monoclonal antibody was labeled with La; anti-CD110—with Eu; anti-CD61—with Dy; anti-CD45—with Ho; anti-CD54—with Nd; CD49d—with Pr. Cells were reacted with labeled antibodies either with each separately, or with all antibodies simultaneously (ALL; 6-plexing). Note that the highly expressed ubiquitous nucleated blood cell marker CD45 (Ho) is on average 10 times greater than cell adhesion markers (CD54, Cd38, CD49d), and 100 times greater than megakaryocite differentiation markers CD61 and CD110 (cells were not induced to differentiate along the megakaryocite pathway).

FIG. **14**. a. Is a three-dimensional bar graph showing the direct comparison of fluorescence obtained from cells stained with CD33-FITC or dual labeled CD33-FITC-Pr using flow cytometry. b. Is a three-dimensional bar graph showing the direct comparison of normalized response obtained from cells stained with CD33-Pr or dual labeled CD33-FITC-Pr using ICP-MS.

FIG. **15** is a flow Chart I of RAFT polymerization procedure.

FIG. **16** is a flow Chart II of Polymer-DTPA-Linker attachment procedure.

DESCRIPTION OF THE VARIOUS EMBODIMENTS

The overall requirements for an element tag are less stringent than those for a fluorescent tag[12] since the chemical nature of an element is not important for its detection by elemental analysis. The tag should contain a reproducible and, preferably, large number of atoms of a given element or isotope composition. The tag can comprise one element or isotope, or consist of a composition of more than one element or isotope. It can also include a natural mixture of isotopes. Further, it is possible that the element tag can comprise one pendant group comprising a certain metal or isotope and a second pendant group comprising another metal or isotope. Reproducibility in the number of identical atoms incorporated is a basis for quantitative analysis, and an increase in the number of those atoms improves the sensitivity linearly. Another key attribute is resistance to leaching, which distinguishes this invention from the DELFIA products. Mobility of the chelated metal is required in the DELFIA products (DELFIA® Assays and Reagents, PerkinElmer, USA). The tag atoms can be any atoms of an element or isotope that differentiate the tag from other atoms in the sample including from other tag atoms associated with differentiated element tags. Typically, the tag atoms will be metals, in particular transition elements, and most typically lanthanides.

The tags to be employed for the detection of analytes have the general structure I of FIG. **1**.

The polymer can be any polymer as is known to those skilled in the art. Examples of polymers are shown in FIGS. **1** through **4**. Further, the polymer backbone can be derived from a substituted polyacrylamide, polymethacrylate, or polymethacrylamide. Further still, the backbone of the polymer can be a substituted derivative of a homopolymer or copolymer of acrylamides, methacrylamides, acrylate esters, methacrylate esters, acrylic acid or methacrylic acid. The polymer can be synthesized by many methods as are known to those skilled in the art. For example, the synthesis can be accomplished with compounds such as N-alkyl acrylamides, N,N-dialkyl acrylamides, N-aryl acrylamides, N-alkyl methacrylamides, N,N-dialkyl methacrylamides, N-aryl methacrylamides, methacrylate esters, acrylate esters and functional equivalents thereof.

The ligand or pendant group can be any ligand as is known to those skilled in the art. Examples of ligands are shown in FIGS. **2** through **4**.

The linker can be any linker as is known to those skilled in the art. Examples of linkers are shown in FIGS. **5** and **6**. The linker is optional.

The spacer is optional. Examples of spacers include PEG block spacers, and others known to those skilled in the art.

The invention involves primarily but not exclusively the following aspects:

(i) Polymeric metal tag carrier synthesis. Functionally, the metal tagged polymer is stable under typical assay conditions, which includes very low kinetic lability of bound metals and rate of exchange of metals between polymers;

(ii) Synthesis and characterization of the attachment (linker) group in combination with polymeric metal tag carrier;

(iii) Synthesis of tagged affinity reagent, which functionally includes an attachment (linker) group in combination with the polymeric metal tag carrier. The tagged affinity reagent can be a tagged antibody or other tagged affinity reagent; and

(iv) Method of employing the affinity reagents as multiplexing tools.

More generally the invention involves synthesis and testing of metal-containing tags for labeling of bio-organic molecules, including affinity reagents such as antibodies. Specifically designed for elemental analysis, such a tag would typically be: (i) water soluble, (ii) non-toxic, (iii) easily separated from a tagged material by known chromatographic, centrifugation, filtration or dialysis methods; and, in addition, can have three or four moieties: the attachment group (linker), possibly a spacer (for example, a PEG spacer), the polymer skeleton (carrier), and the tag atoms (as many tag atoms (of the same metal or isotope, or of a different metal and/or isotope) as possible). For different elemental analyzers the characteristics of the element tag can be similar.

Although an embodiment of the invention using antibodies as the affinity reagent is exemplified, it is to be understood that other affinity reagents can be used and are within the scope of the invention.

Polymer carrier: An important aspect of the invention is the synthesis of a polymer, to which a large number of tag atoms can be attached. Typically the tag atoms are metal atoms. The polymer can be water soluble This moiety is not limited by chemical content. However, it simplifies analysis if the skeleton has a relatively reproducible size (for example, length, number of tag atoms, reproducible dendrimer character, etc.). The requirements for stability, solubility, and non-toxicity are also taken into consideration. Thus, the preparation and characterization of a functional water-soluble polymer by a synthetic strategy that places many functional groups along the backbone plus a different group at one end that can be used to attach the polymer via a linker to a biomolecule (for example, an affinity reagent) is part of this invention.

The tags to be employed for the detection of analytes have the general structure I of FIG. **1**. The signal to be detected will be that of the polymer, which will contain between approximately 1 to 1000 (or more) atoms of an element (for example, lanthanide (Ln) atoms) as part of its structure. A flexible linker/spacer at one end of the polymer may contain a thiol-reactive functional group such as a maleimide, and through this group can be linked to an affinity reagent (for example an antibody) for the specific target analyte. Variations include the attachment to primary amines of biomolecules or other methods of attachment known to persons skilled in the art. Examples of the selection of functional groups for the linker arm can be taken from the literature on PEGylated antibodies, reviewed recently by Chapman[13]. The polymers as carriers of the metal-atom tags have a similar number of backbone atoms as those of the PEG polymers that have been attached to various antibodies without loss of binding affinities. For example a PEG2000 (2 KDa) has a mean degree of polymerization of 45 corresponding to 140 backbone atoms, and PEG5000 has 340 backbone atoms. To put these tags in perspective, the average size of an IgG antibody from the end of the Fc to the Fab is approximately 11 nm[14]. The radius of gyration of the polymer constructs should be as small as possible, somewhere between approximately 2 nM and 11 nM.

In one embodiment, the invention involves, polymers containing the Ln3+ atoms as substituents of the pendant groups and their synthesis. In structure "a" of FIG. **1**, each repeat unit of the polymer bears the liganded Ln3+, the

group being denoted by (L). It is neither likely nor required that each pendant group bear an (L) substituent. In structure b of FIG. **1**, a fraction of the repeat units have an organic group R. In these structures, the asterisk (*) represents the initiated end of the polymer. The following factors are considered: 1) The polymer can be water soluble. Because of their hydrolytic stability, N-alkyl acrylamides, N-alkyl methacrylamides, and methacrylate esters or functional equivalents can be used. 2) A degree of polymerization (DP) of approximately 1 to 1000 (1 to 2000 backbone atoms) encompasses most of the polymers of interest. Larger polymers are in the scope of the invention with the same functionality and are possible as would be understood by practitioners skilled in the art. Typically the degree of polymerization will be between 10 and 250. 3) The polymers may be amenable to synthesis by a route that leads to a relatively narrow polydispersity. The polymer may be synthesized by atom transfer radical polymerization (ATRP) or reversible addition-fragmentation (RAFT) polymerization, which should lead to values of Mw/Mn in the range of 1.1 to 1.2. An alternative strategy involving anionic polymerization, where polymers with Mw/Mn of approximately 1.02 to 1.05 are obtainable. Both methods permit control over end groups, through a choice of initiating or terminating agents. This allows synthesizing polymers to which the linker can be attached. 4) A strategy of preparing polymers containing functional pendant groups in the repeat unit to which the liganded transition metal unit (for example a Ln unit) can be attached in a later step can be adopted. This embodiment has several advantages. It avoids complications that might arise from carrying out polymerizations of ligand-containing monomers. In addition, the polymer backbone is a known one that can be adapted for most if not all of the Ln-containing polymers. Thus the polymers may have a common mean chain length and chain-length distribution. 5) The target polymers of type "a" may either be negatively charged polyelectrolytes or have zwitterionic pendant groups. To minimize charge repulsion between pendant groups, the target ligands for (Ln3+) should confer a net charge of −1 on the chelate. For type "b" polymers, the R groups are for the most part uncharged, although in one example, the inventors teach a polymer in which the small fraction x of R groups will have a positive charge. Finally, various chemistries are well known that enable the attachment of the linker group with its thiol reactive group to the polymer. A number of pendant groups can be added to the polymer. Practically, the number can be between 1 and 1000, and more typically between 10 and 250. The metal-binding pendant group can be attached to the polymer by methods known to those skilled in the art, for example, the pendant group may be attached through an ester or through an amide.

Examples for the synthesis of functional ligands that are used to attach (L) to the polymer are shown in FIG. **2** (Schemes 1 and 2). The examples are exemplary and are not intended to limit the scope of the invention.

Chelate (tag atom) choice and synthesis: The use of the lanthanides is established here as feasible, however, similar results can be achieved for different elements. Across the series of lanthanides very similar coordination chemistry is observed. All the lanthanides favor the +3 oxidation state and preferentially coordinate with hard oxygen ligands. Lanthanides do not exhibit defined coordination geometries and vary in coordination number between 7 and 10 across the series. Thus, the same chelate-derivatized polymer can be used for all the Ln metals, which facilitates production of tags containing different lanthanides used in multiplexing assays[15]. Different embodiments utilizing different metals can be obtained using similar considerations related to their chemical nature. Numerous Ln complexes have been developed for use as radiopharmaceuticals and imaging agents[16]. But the art does not disclose metal atoms attached to pendant groups on the polymer backbone. The multidentate chelates developed for these applications form thermodynamically stable and kinetically inert Ln complexes, important for minimizing the toxicity of free lanthanides in vivo. Incorporating these optimized lanthanide chelates, as pendant groups on polymeric structures, appears to be described here for the first time.

As examples, two ligand frameworks as functional examples of covalently linked chelates on the polymeric backbone are described. The selection criteria for this embodiment include known syntheses, heptadentate or octadentate coordination to promote kinetic stability against metal ion dissociation, a pendant primary amine functional group for attachment of the chelate to the polymer, and a net charge of −1 for the liganded chelate. Diethylenetriaminepentaacetate (DTPA), an acyclic chelator can be readily derivatized as an amine functionalized ligand (Scheme 1, FIG. **2**). Coupling a monoprotected diamine with the commercially available DTPA anhydride, followed by deprotection provides a candidate ligand to be coupled to the polymeric active ester. The net charge of the compound once complexed to lanthanide is −1. The facile synthesis of this chelator makes it an attractive starting point for optimizing the polymeric backbone with attached chelators.

DTPA ligands are inherently more kinetically labile than the macrocyclic ligand based on the cyclen framework. The macrocyclic nature of the cyclen-based ligands preorganizes the metal binding site, leading to greater thermodynamic and kinetic stability. These chelates are known to be stable in vivo for days[17]. Reaction of commercially available tritert-butylmethylcyclen (Macrocylics) with the readily available homoserine derivative provides an orthogonally protected DOTA derivative (Scheme 2, FIG. **2**)[18]. The Fmoc protecting group can be removed to access the amine and make it available to couple with the polymeric backbone. In some instances it may be necessary to employ a spacer between the DOTA chelate and the polymer. A variety of selectively protected amino acids of different lengths is commercially available and can be readily coupled and deprotected to form linkers. The lanthanide complex of this chelate will carry a net −1 charge. Based on functionality, these Ln chelates with the reactive —NH_2 group are referred to as (L)-NH_2.

Polymer synthesis and chelate attachment: Herein below, the synthesis of candidate polymers, the attachment of functional chelates to the polymer backbone, and the characterization of the metal containing polymers are described. These are intended to be examples, and not to limit the scope of the claims. Other examples can be used as is known to those skilled in the art.

Random copolymer poly(DMA-co-NAS): A recent report[19] describes the synthesis of a 75/25 mole ratio random copolymer (3, FIG. **3**) of N-acryloxysuccinimide (NAS) with N,N-dimethyl acrylamide (DMA) by RAFT with high conversion, excellent molar mass control in the range of 5000 to 130,000, and with Mw/Mn≈1.1. In this embodiment (Scheme 3, FIG. **3**), the active NHS ester of 3, FIG. **3** is reacted with a liganded lanthanide (L) bearing a reactive amino group to yield the copolymer 4, FIG. **3**. FIG. **15** is a flow chart showing the steps involved in RAFT polymerization.

Poly(NMAS): Yet another approach has been reported by Müller[20] and used to attach drug conjugates to the polymer backbone. In this approach, Müller polymerized NMAS by

ATRP (Scheme 4, FIG. **3**), obtaining polymers with a mean molar mass ranging from 12 to 40 KDa with Mw/Mn of approximately 1.1. In their experiments, limiting amounts of various drugs or drug-mimics bearing a spacer and a primary amine were reacted with the NHS ester groups of 5, FIG. **3**, and then the remaining sites were reacted with excess Me_2NH. Their initiator was the hydroxyethyl ester of bromoisobutyric acid; thus the polymer chains all had a primary alcohol as an end group. Here samples of 5, FIG. **3**, are reacted with excess (L)-NH_2, maximizing the number of (L) groups that can be attached to the polymer.

Poly(MAA): Another aspect of the Applicant's teaching is related to specific functional advantages of polymer tags with a very narrow molar mass distribution. Polymethacrylic acid (PMAA) can be prepared by anionic polymerization of its t-butyl or trimethylsilyl (TMS) ester. If the reaction is terminated with ethylene oxide prior to ester hydrolysis (FIG. **4**), the polymer will bear a —CH_2CH_2—OH as a functional end group. A route for attaching (L) to the polymer involves reacting the tetrabutylammonium carboxylate salt of the polymer with the bromoacetamide derivative of (L)-NH_2 (Scheme 5, FIG. **4**).

Poly(DMAEMA): Recently, samples of poly(dimethylaminoethyl methacrylate) (PDMAEMA) were prepared by ATRP[21]. This is a well-known polymer that is conveniently prepared with mean Mn values ranging from 2 to 35 KDa with Mw/Mn of approximately 1.2 This polymer can also be synthesized by anionic polymerization with a narrower size distribution[22]. This polymer can be reacted with the bromoacetamide derivative of (L)-NH2. This yields a zwitterionic polymer 8, Scheme 6, FIG. **4**, which has suitable water solubility. The unreacted dimethylaminoethyl groups will be protonated at neutral pH and contribute a small positive charge to the polymer.

Spacers: A potential source of interference between a metal-bearing polymer tag and affinity reagent activity is the close proximity of the bulky polymer when attached to the affinity reagent. Spacers, for example, PEG spacers, can be situated between the linker and the polymer or between the polymer and the linker. Methods for the addition of spacers is known to those skilled in the art.

The spacer can also be an integral part of the polymer backbone to help mitigate this problem. In the applicant's teaching, the syntheses (for example see Schemes 4-6, FIGS. **3** and **4**) can be modified to create PEG block copolymers. The PEG portion of the block copolymer serves as a PEG spacer, and the synthetic strategies make it possible to vary the PEG spacer length as needed in response to bioassay results that indicate problems with binding efficiency or sensitivity. The spacer can be any spacer as is known to those skilled in the art. For example, it can be a minimal spacer as shown in Scheme 12 and compound 12. This specific enactment seems to be novel as we are not aware of its prior application.

End-group control and coupling chemistry: According to the Chapman review on PEGylated antibodies[13], approaches to PEG attachment via reaction with the free amino group of the lysine were successful, but the PEGylated antibodies obtained exhibited reduced antigen binding efficiency. It appears that the random nature of the chemical reaction to the various lysine groups in the antibody led to PEG attachment at sites that interfered with binding. A more benign result was obtained for the case in which the PEG chain was attached specifically to a single cysteine in the FC fragment that was introduced into the antibody through site-specific mutation. Here reduction of a disulfide bond within the FC fragment of the antibody, followed by covalent attachment of the polymers to one or both of the —SH groups formed is described. Thus a thiol reactive group may be used at one terminus of the polymers.

RAFT polymers: The thiobenzoate end group of RAFT polymers is conveniently converted to a terminal —SH group. This chemistry is shown in scheme 7a, FIG. **5**, for polymer 4, FIG. **3**. Numerous methods are known, to those skilled in the art, for crosslinking thiols, in analogy with reactions described for —SH terminated polyethylene glycol (PEG-SH)[23], and allow the attachment of the polymer via the mixed disulfide to the free —SH of an antibody or other affinity reagent (denoted as "protein-SH"). Alternatively, bismaleimide derivatives are commercially available and alkylation of the polymer with these reagents followed by GPC (Gel Permeation Chromatography) purification and reaction with the free thiol of the antibody or other affinity reagent provides the desired conjugate[24].

ATRP polymers: Polymers of the structure 5, FIG. **3**, reported by the Müller group[25] have a terminal —CH2CH2-OH group. A different initiator for the polymerization reaction is described here. 2,6-napthalene derivatives are readily available and will provide an orthogonally protected amine. After deprotection, reaction of the amine with a bifunctional NHS-maleimide, the thiol-amine cross-linking agent will provide the polymeric labeling agent for antibody conjugation. This initiator also provides a convenient chromophore for quantification of the polymer. This also shown in scheme 7b in FIG. **5**.

Anionic Polymerization (Scheme 5, FIG. **4**): Anionic polymerizations can often be terminated by reaction with functional electrophiles to introduce an end group to the polymer[26]. Enolates react effectively with allylic and benzylic halides[27]. Quenching styrene polymerization with epichlorohydrin has been shown to be problematic[28]. Conditions for quenching the enolate end of a living poly(t-butyl methacrylate) to yield the terminal epoxide are described here. While glycidyl methacrylate can be polymerized anionically at low temperature in the presence of LiCl, which makes the propagating anion less nucleophilic[29], it is expected that the enolate of t-butyl methacrylate should ring-open an epoxy group at higher temperature[30]. Opening of the epoxide with azide provides an orthogonal functional group stable under conditions of ester hydrolysis. Treatment of azides with an alkyne in the presence of Cu(I) salts yields triazoles in high yield[31]. By using this coupling reaction a thiol reactive maleimide is installed at the terminus of the polymer. This is also shown in scheme 7c in FIG. **5**.

Attachment (linker) groups: The attachment group provides a covalent bond between bioorganic (proteins, peptides, oligonucleotides) molecules, for example affinity reagents, and the element tag. For example, the linkage can be effected via thiols using a maleimido attachment group; through the N-terminus or basic side chain (lysine, arginine, histidine) (see Scheme 8c, FIG. **6**), through the C-terminus or acidic side chain (aspartic acid, glutamic acid) using p-(chloromethyl)styrene (see Scheme 8c, FIG. **6**), or via oxidation of the sugar moiety on the antibody or other affinity reagent and coupling via a hydrazine group. One may take advantage of thiol groups created by reduction of the disulfide bond in the FC fragment of the antibody. This combination "bioorganic molecule—attachment group—element tag" is thought to be described here for the first time.

Functional example of coupling chemistry: There are four main coupling chemistries commonly used to attach polymers (such as PEG) to the free thiols of proteins. The advantages and disadvantages of each of these reactions have recently been reviewed[32]. One approach involves dis-

ulfide exchange as shown in Scheme 7a, FIG. **5**. Three other common reactions involve addition of —SH to a maleimide or a vinyl sulfone and the displacement of iodide from an iodoacetamide (Schemes 8a-c, FIG. **6**). To avoid the slow hydrolysis in water that is typical of maleimide and iodoacetamide groups, a strategy in which the thiol-reactive agent is added to the end of the (L)-bearing polymer just prior to tagging of the affinity reagent is possible. This strategy takes advantage of the "click" chemistry developed recently by Sharpless[33] (Scheme 8b, FIG. **6**) involving the 1,3-dipolar addition of azides to acetylenes, a reaction that Sharpless has shown to occur under mild conditions with quantitative yield. To introduce the acetylene unit on the end of polymers bearing a terminal —NH_2 group, they are reacted with an active ester derivative of 4-pentynoic acid. The polymer is then set up for a reaction with a derivative of the form X—R—N3, where R is the spacer and X represents the thiol-reactive group.

Coupling of polymer to an antibody or other affinity reagent: As an example, reduction of disulfide bonds in an antibody or other affinity reagent can be performed using immobilized trialkylphosphine TCEP (Tris[2-carboxyethyl] phosphine hydrochloride) covalently linked to a beaded agarose support (Pierce). TCEP is known to be an efficient reductant of alkyl disulfides over a wide range of pH and does not interfere with commonly used sulfhydryl-reactive reagents such as maleimide cross-linkers. The use of beads permits recovery of the reduced antibody or other affinity reagent by simple centrifugation from the reducing agent with subsequent separation from the beads.

Purification of polymer modified antibodies: Due to the large size of the IgG antibodies (150 KDa) one option is to separate the excess metallated labeling polymer (20-40 KDa) from the antibody using gel filtration chromatography. Alternatively, Protein A and Protein G have been used to purify antibodies.

As is known to those skilled in the art, the element or metal atoms can be added to the polymer tag at different steps during the production of the tagged biomolecule. It is beneficial to add the element (metal) of the tag after conjugation of the antibody or other affinity reagent with the ligand-polymer. This strategy has several advantages: i) conversion of antibody-ligand-polymer conjugate into antibody-metal-polymer conjugate can be done directly before bio-assay; ii) the multitude of affinity molecules can be tagged with the same ligand-polymer conjugate under the same conditions. The choice of metal (or isotope) to use can be determined directly before the multiplexed experiment by the reagent user significantly increasing experimental flexibility; iii) decoupling of both tagging stages allows series of important independent control experiments in which the same antibody can be tagged with different metals; iv) selection of the internal standards is unhindered, and the relative sensitivity of the elemental analyzer can be effectively controlled.

The order of steps for the synthesis of the tagged biomolecule can take many forms. Three examples are provided below, but it is to be understood that other orders of steps are possible:

A	B	C
Synthesize polymer	Synthesize polymer	Synthesize polymer
Bind metal to polymer	Bind linker to polymer	Bind linker to polymer
Bind linker to polymer	Bind metal to polymer	Bind linker to antibody
Bind linker to antibody	Bind linker to antibody	Bind metal to polymer

Further, the linker can be attached to the biomolecule before the linker is attached to the polymer. Most often, the metals will be attached anytime before binding the tagged affinity reagent to the analyte. It is possible to add the metals after attaching the affinity reagent to the analyte, but the background is expected to be elevated because many analytes, and in particular cells, will bind metals non-specifically. It is therefore less likely to be performed successfully after binding the affinity reagent to the analyte.

Further, the polymer element tag may be attached to a biomolecule which is other than an affinity reagent. For example, the polymer element tag may be attached directly to an analyte, for example but not limited to a growth factor, cytokine or chemokine for studying kinetics of ligand-receptor interactions. Specifically, EGF (epidermal growth factor) with polymer element tag may be used as a probe to investigate EGFR (epidermal growth factor receptor) abundance on cell surface, receptor dimerization and internalization. This aspect is also within the scope of the applicant's teachings. Two or more analytes may also be analyzed in a multiplex reaction.

Aspects of the Applicant's teachings may be further understood in light of the following examples, which should not be construed as limiting the scope of the present teachings in any way.

EXAMPLES

Example 1. Synthesis of Copolymers of N,N-Dimethylacrylamide and N-Acryloxysuccinimide by RAFT Polymerization

N,N-dimethylacrylamide (DMA) and N-acryloxysuccinimide (NAS) were copolymerized by the reversible addition-fragmentation chain transfer (RAFT) polymerization technique, to obtain random copolymer precursors with side-groups statistically grafted via the reactive NAS units[19]. The random copolymers of DMA and NAS, poly(DMA-co-NAS), were prepared using tert-butyl dithiobenzoate (t-BDB) as chain transfer agent (CTA) (Scheme 9, FIG. **7**).

Preparation of tert-Butyl Dithiobenzoate (t-BDB).[34] In a 500 mL round-bottomed flask equipped with a magnetic stirrer, 150 mL of a diethyl ether solution of s-(thiobenzoyl) thioglycolic acid (0.27 g, 2.4 mmol) was added to 100 mL of an aqueous basic solution (NaOH, 1 mol L^{-1}) of sodium 2-methyl-2-propanethiolate (0.510 g, 2.9 mmol). This biphasic mixture was vigorously stirred at room temperature for 5 hours. Then, the purple ether phase was removed and washed twice with 500 mL of an aqueous basic solution (NaOH 1 mol L^{-1}) and twice with 500 mL of a 10% NaCl aqueous solution and dried over anhydrous magnesium sulfate. Purification by silica gel chromatography (Kiesegel-60) with petroleum ether/ethyl acetate (99/1:v/v) as eluent gave tert-butyl dithiobenzoate (t-BDB) as a dark purple oil (90% yield). 1H NMR (CDCL3) d (ppm): 1.69 (S, 9H, 3×CH3), 7.36 (m, 2H, meta-ArH), 7.50 (m, 1H, para-ArH) and 7.88 (m, 2H, ortho-ArH).

Preparation of N-acryloxysuccinimide (NAS).[35] N-hydroxysuccinimide (10 g, 0.086 mol) and triethylamine (13.2 g, 0.129 mol) were dissolved in chloroform (130 mL) at 0° C. Acryloyl chloride (8.6 g, 0.094 mol) was added dropwise over a period of 2 hours to the stirred reaction mixture. The reaction is described in Scheme 1, FIG. **2**. After being stirred an additional 30 minutes at 0° C., the solution was washed twice with 60 mL saturated NaCl aqueous solution, dried over MgSO4, filtered and concentrated so as to get a residual volume of 30 mL. An ethyl acetate/pentane mixture (14 mL,

1:3 v/v) was added and the temperature was maintained at 0° C. to induce NAS crystallization overnight (70% yield). 1H NMR (CDCl3) d (ppm): 2.95 (S, 4H, CH2CH2), 6.20 (m, 1H, CH═CH2), 6.4 (m, 1H, CH═CH2) and 6.75 (m, 1H, CH═CH2).

Preparation of random copolymers of DMA and NAS. General experimental conditions: DMA was distilled under reduced pressure prior to use. Monomers, t-BDB, initiator 2,2'-azobis(2-methylbutyronitrile) (AMBN) and solvent dioxane were introduced in a schlenk tube equipped with a magnetic stirrer. The mixture was degassed by three freeze-vacuum-thaw cycles and then heated under argon in a thermostated oil bath at 80° C. The percentage yields were calculated gravimetrically.

The structure of copolymers has been verified by application of appropriate chromatographic and spectrometric methods. Gel permeation chromatography (GPC) has been used to establish the molecular weight and molecular weight distribution of the copolymers. A Viscotek liquid chromatograph equipped with a Viscotek VE3210 UV/vis detector and a VE3580 reflective index detector and Viscotek GMHHR-M Viscogel™ GPC column was used. The flow rate was maintained at 0.5 mL min-1 using a Viscotek VE1121 GPC pump. 1-Methyl-2-pyrrolidinone was used as eluent. The molecular weights are provided as polystyrene equivalents. FIG. **15** is a flow chart of the RAFT polymerization procedure.

Preparation of copolymer containing 13 mol % of NAS units. NAS (0.81 g, 4.82 mmol), DMA (3.2 mL, 31 mmol), AMBN (70 mg, 0.36 mmol) and t-BDB (116 mg, 0.521 mmol) were added into 33 mL of 1,4-dioxane. The solution in a schlenk tube was degassed and heated at 80° C. for 18 hours. Then the solution was cooled and precipitated in 300 mL diethyl ether. The collected solid was redissolved in 1,4-dioxane and precipitated in diethyl ether. Yield of dried polymer was 75%. The molecular weight and polydispersity are shown in FIG. **8**.

Preparation of copolymer containing 47 mol % of NAS units. NAS (2.33 g, 13.9 mmol), DMA (N,N-dimethylacrylamide 1.6 mL, 15.5 mmol), AMBN (70 mg, 0.36 mmol) and t-BDB (116 mg, 0.521 mmol) were added into 30 mL of 1,4-dioxane. The solution in a schlenk tube was degassed and heated at 80° C. for 18 hours. Some precipitation was observed in the tube. Then the solution was cooled and precipitated in 300 mL diethyl ether. The collected solid was redissolved in DMF and precipitated in diethyl ether. Yield of dried polymer was 80%. The molecular weight and polydispersity are shown in FIG. **8**.

Preparation of copolymer containing 60 mol % of NAS units. It was prepared by a similar procedure as aforementioned (47 mol % of NAS units) one. More NAS monomer was added and solvent 1,4-dioxane was substituted by DMF. Yield of dried polymer was 80%. The molecular weight Mn and polydispersity Mw/Mn are shown in FIG. **8**.

Example 2. Preparation of Ligand-Polymer Conjugate

The following preparation of the polymer ligand conjugate is amenable for use with any amine functionalized ligand according to Scheme 10 and Scheme 11, FIG. **9**.

To a stirred solution of the (N,N-dimethylacrylamide (DMA) and N-acryloxysuccinimide (NAS)) copolymer containing 47 mol % of NAS units (35 mg, 3.5461 mmol) and N,N-diisopropylethylamine (300 μl) in DMF/H_2O (60:40, 1 mL) was added a solution of the amine pendant ligand 9, FIG. **9** (78 mg) in the same mixture (2 ml). The reaction mixture was stirred overnight under nitrogen at room temperature. The solvent was removed under vacuum and the solids were dissolved in H_2O. The solutions were dialyzed by repeated washings with deionized water (5×4 mL) in an Amicon centrifugal filter (5K MW C.O.) The solution remaining in the filter device was concentrated to give a yellowish solid. The solid was purified further by precipitation from methanol with diethylether to give a yellow powder (48 mg)

Ligand-polymer conjugate (5.5 mg) was dissolved in 1 mL of 50 mM phosphate buffer, (pH 8.5. 2 mL of 20 mM DTT) and the reaction mixture was stirred for 1 hour at 50° C. After the reaction, the mixture was made acidic (pH 4) with acetic acid and washed in an Amicon centrifugal filter (5K MW C.O.) with aqueous acetic acid (5 mM, 5×4 mL). The solution left in the filter device was then transferred to a small reaction flask containing 2 mL of 100 mM phosphate buffer, pH 8.5. A solution of 1,4-bis(maleimido)butane (50 equiv.; 32 mg) in DMF was added to the flask and the reaction mixture was stirred overnight at 50° C. The solvent was evaporated to give a residue, which was dissolved in H_2O, and the clear solution was again washed using an Amicon centrifugal filter (5K MW C.O.) with deionized water (5×5 mL). The supernatant was lyophilized to give the final conjugated polymer (4 mg).

Example 3. Preparation of Ligand-Polymer Conjugate: DOTA Based Conjugate According to Scheme 12, FIG. **10**

To a stirred solution of the DMA-NAS copolymer with 60 mol % of NAS units (100 mg) in DMF (3 mL) and triethylamine (1 mL) was added a solution of amine pendant ligand 10 (363 mg, 0.590 mmol) in DMF (2 mL). The reaction mixture was stirred overnight under nitrogen at room temperature. After the solvent was removed under vacuum, the residue 11 was dissolved in neat trifluoroacetic acid (3 mL) and stirred overnight at room temperature. The solution was evaporated, and the residue was taken up in water and dialyzed by repeated washings with deionized water (6×5 ml) in an Amicon centrifugal filter (5K MW C. O.). The solution remaining in the filter device (ca. 0.8 mL) was concentrated to give a yellow solid 12 (179 mg).

The entire sample of polymer-ligand conjugate 12 was dissolved in 50 mM phosphate buffer (pH 8.5, 2 mL) containing 20 mM DL-dithiothreitol, and the reaction mixture was stirred for 1 hour at 50° C. After this time, the mixture was acidified to pH 4 with acetic acid, and washed in an Amicon centrifugal filter (5 K MW C. O.) with aqueous acetic acid (5 mM, 5×5 mL). The solution left in the filter device (0.8 mL) was then transferred to a small reaction flask containing phosphate buffer (100 mM, pH 8.5, 5 mL). A solution of 2,2'-(Ethylenedioxy)bis(ethylmaleimide) (191 mg, 0.619 mmol) in DMF (2 mL) was added to the flask and the reaction mixture was stirred for 1 hour at room temperature. Water (3 mL) was added into the flask and the solid was removed by filtration. The resulting clear solution was again washed with deionized water (5×5 mL) using an Amicon centrifugal filter (5K MW C. O) and the supernatant was purified by Sephadex G-50 Column with HPLC system using water as an eluent. The fraction was collected and lyophilized to give the final conjugated polymer 13 (165.0 mg).

Example 4. Preparation of Ligand-Polymer Conjugate: DTPA Based Conjugate According to FIGS. **9**, **11** and FIG. **16**

To a stirred solution of the DMA-NAS copolymer with 60 mol % of NAS units (2.0 g) in DMF (30 mL) and triethyl-

amine (4.3 mL) was added a solution of tert-butyl 2-aminoethylcarbamate, 14 (2.5 g, 15.6 mmol) in DMF (10 mL). The reaction mixture was stirred overnight under nitrogen at room temperature. Then the mixture was precipitated in 500 mL of diethyl ether. The collected solid 15 (400 mg) was dissolved in neat trifluoroacetic acid (3 mL) and stirred overnight at room temperature. The solution was evaporated, and the residue was taken up in water and dialyzed by repeated washings with deionized water (6×5 ml) in an Amicon centrifugal filter (5K MW C. O.). The solution remaining in the filter device (ca. 0.8 mL) was concentrated to give a yellow solid 16 (210 mg).

DTPA succinimidic ester was prepared according to a published procedure.[36] 16 g of DTPA (40.64 mmol) dissolved in 320 mL of Acetonitrile (23 g, 230 mmol of triethylamine added). Solution was stirred at 50° C. to dissolve the DTPA. 3.36 g of dicyclohexylcarbodiimide (DCC, 16.3 mmol) and 1.9 g of N-Hydroxysuccinimide (NHS, 16.3 mmol) were added simultaneously at room temperature. The reaction was carried out overnight. White precipitate was observed and filtered off by filtration paper, generating solution (A).

210 mg of solid 16 (ca. 0.8 mmol amino groups) was dissolved in 80 mL of distilled water and added into solution (A) at room temperature. 5 mL of triethylamine was added, and the solution was stirred at room temperature overnight. Solvents (triethylamine, acetonitrile) was then evaporated and 100 mL water added. The solution was dialyzed (1K cut-off membrane) for two days. Then the aqueous solution was concentrated, and acetic acid was added. It was dialyzed again with the same membrane for another three days. The solution is concentrated to give a solid 17 (190 mg).

Solid 17 (110 mg) was dissolved in 2.3 mL of phosphate buffer solution (pH 7.2). Then tri(2-carboxyethyl)phosphine (TCEP, 0.18 mL of 0.5 M solution) was added into buffer solution at room temperature. After the solution was stirred for 2 hours, it was added into 2,2'-(ethylenedioxy)-bis(ethylmaleimide) (0.36 mmol, 106 mg) in 2.3 mL of DMF at room temperature. 100 mL of distilled water was added after 2 hours and the solution was filtered through 5 k cut-off membrane with 5% DMSO/water (2 times) and then distilled water (3 times). The fraction was collected and lyophilized to give the final conjugated polymer 18 (90 mg). FIG. **16** is a flow chart showing the procedure for polymer-DTPA-linker attachment procedures.

Example 5. Preparation of Ligand-Polymer Conjugate: Poly(MAA) or Poly(AA)

One aspect of the invention is related to specific functional advantages of polymer tags with a very narrow molar mass distribution. Polymethacrylic acid [Poly(MAA)] or polyacrylic acid [Poly(AA)] can be prepared by anionic polymerization of its t-butyl or trimethylsilyl (TMS) ester.[37] If the reaction is terminated with tert-butyldimethylsilyl 3-chloropropyl sulfide,[38] prior to ester hydrolysis (see below), the polymer will bear a protected —SH functional end group. They are reacted with tert-butyl 2-aminoethylcarbamate to form a polymer with protected amino groups, which is then hydrolyzed into polymer 19 (FIG. **12**, Scheme 13). The free amino groups on main chain of polymer 19 offer sites for chelate attachment. The route for attaching chelate refers to the previous procedure using DTPA succinimidic ester (FIG. **11**).

Poly(NMAS). Another approach has been reported by Müller[39] and used to attach drug conjugates to the polymer backbone. In this approach, NMAS was polymerized by ATRP, obtaining polymers with a mean molar mass ranging from 12 to 40 KDa with Mw/Mn of approximately 1.1. The initiator used was the hydroxyethyl ester of bromoisobutyric acid; thus the polymer chains all had a primary alcohol as an end group. Here, a new initiator based on cystamine 20 can be prepared (FIG. **12**, Scheme 14). It is then used in the ATRP of NMAS to form a polymer 21 (FIG. **12**, Scheme 14) with disulfide group. The polymer 21 can be reacted with tert-butyl 2-aminoethylcarbamate as shown in FIG. **12**, Scheme 13 to generate a polymer with pendent amino groups for attachment of DTPA ligands. By using tri(2-carboxyethyl)phosphine (TCEP), the disulfide bond was reduced and a thiol end-group was generated for attachment of a linker to an antibody (FIG. **12**, Scheme 15).

Example 6. Multiplex Labeling of Leukemia Cells

K562 cells, a model cell line of human chronic myeloid leukemia, were cultured under standard tissue culture conditions in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS (fetal bovine serum), 2 mM L-glutamine, and antibiotics. Growing cells were collected by low speed centrifugation (500×g), washed once with phosphate buffered saline (PBS), pH 7.4 and immunolabeled with primary antibodies attached to the metal-polymer conjugate (Ho, Dy, Nd, Eu, Pr, or La separately for each antibody) as described in Scheme 10 and Scheme 11 (FIG. **9**). Six cell surface-specific antibodies were chosen for the experiment: CD38, CD110, CD61, CD45, CD54, CD49d. Aliquots of cells in triplicate tubes (0.3×106) were labeled with each antibody separately or with all antibodies combined in one reaction mixture (sample ALL). As negative control, mouse IgG1 isotype immunoglobulins were attached to metal-polymer conjugates carrying the same elements as the primary antibodies—Ho, Dy, Nd, Eu, Pr, or La. After 30 minutes incubation on ice, the cells were washed with PBS three times by centrifugation. The final cell pellet was dissolved in concentrated HCl (35%), mixed with an equal volume of 1 ppb Ir/HCl solution as internal standard and subjected to volume analysis ICP-MS. Results are presented in FIG. **13**.

Antibodies were attached to the metal-polymer conjugate (synthesized according to Scheme 10 and Scheme 11, FIG. **9**) according to the following protocol and reagents.

Reagents:

Antibody at least 100-150 μg (~1 nmol) in 100 μl PBS/EDTA (~1 mg/ml). The antibodies were purchased commercially from BD Biosciences, San Jose, Calif.).

TCEP disulfide reducing gel (4% cross-linked agarose beads) from Pierce #77712; supplied as 50% slurry. Used at 1:1 50% slurry to antibody v/v.

Ligand-Polymer Conjugate (see Scheme 11, FIG. **9**) dissolved in double distilled water (ddH_2O). Expected MW 11,000.

R-Buffer is 0.1M sodium phosphate pH 7.2, 2.5 mM EDTA

C-Buffer is TBS, 1 mM EDTA

L-Buffer is 20 mM ammonium acetate pH 6.0

Reduction of IgG Disulfide Bonds:

Added 200 μL R-Buffer and 50 μg antibody solution to Diafiltration Membrane.

Centrifuged 10,000 g for 10 minutes. Discarded flow-through. Repeated once.

Added 100 μL R-Buffer and 0.8 μL 0.5M TCEP solution to Diafiltration Membrane and mixed gently (4 mM TCEP). Did not vortex.

Incubated 30 minutes at 37° C.

Added 200 μL C-Buffer. Centrifuged 10,000 g for 10 minutes. Discarded flow-through.

Labelling of Reduced IgG:

Added 200 μL C-Buffer to membrane.

Prepared the element tag in C-Buffer at a concentration of 1 mM (1.1 mg element tag in 50 μL C-Buffer).

Added 10 μL of the prepared element tag to the tube containing 200 μL of the reduced IgG solution and mixed well. Did not vortex.

Allowed the reaction to proceed at least 1 hour at 37° C.

Added 200 μL L-Buffer to Membrane. Centrifuged 10,000 g for 10 minutes.

Discarded flow-through. Repeated twice.

Added 100 μL L-Buffer to membrane to resuspend labelled antibody.

Added 5 μL of 0.1M lanthanide solution (prepared in Ultrapure Water as is known to those skilled in the art) to the antibody conjugated with the polymer tag. Mixed well. Did not vortex.

Incubated 30-60 minutes at 37° C.

Added 300 μL TBS. Centrifuged 10,000 g for 10 minutes. Discarded flow-through. Repeated three times.

Added 50 μL TBS. Gently pipetted several times to recover the conjugate and transferred to eppendorf tube.

Although ICP-MS was used in this analysis, it is to be understood that other forms of elemental analysis could have been used and are encompassed in the scope of the applicant's teachings.

Further, although leukemia cells were targeted as the analyte it is understood that any cell or particle can be analyzed in a similar manner.

Example 7. Analysis of Double Labeled Antibodies—Fluorescent Label and Element Tag

In this example, the double labeled antibodies facilitate presorting and subsequent elemental analysis of rare cells in mixed samples by ICP-MS-based flow cytometry.

In one instance demonstration of data congruence collected by flow cytometry (FACS) and ICP-MS of cells stained with dually labelled antibodies (CD33-FITC-Pr) was conducted.

Monoclonal antibodies against cell surface antigen CD33 conjugated to fluoresceneisothiocyonate (FITC) (CD33-FITC; GenTex Inc.) were tagged with the polymer-DOTA-Pr construct. This dual labelled antibody will further be referred to as CD33-FITC-Pr. Several well characterized human leukemia cell lines (KG1a, THP-1, Kasumi-3; ATCC Inc) were used in cell staining studies. FACS analysis was performed on FACScalibur™ flow cytometer instrument (BD Biosciences Inc.) and ICP-MS data was obtained using ELAN DRCPlus (Perkin Elmer SCIEX). Live cells were washed by low speed centrifugation and incubated with CD33-FITC-Pr or CD33-FITC or CD33-Pr for antigen expression controls. Non-specific immunoglobulin binding was monitored with mouse IgG-FITC, IgG-Pr or dual labelled IgG-FITC-Pr. Data presented in FIG. **14***a* shows that fluorescence obtained from cells stained with dual labelled CD33-FITC-Pr are similar to CD33-FITC on all cell lines tested. Note that the KG1a cell line does not express CD33.

Likewise when CD33 expression was tested using element tagged antibodies CD33-Pr and dual labeled CD33-FITC-Pr (FIG. **14***b*), the normalized responses were similar.

Example 8 Particle Elemental Analysis Using a Mass Spectrometer Based Flow Cytometer

The metal-polymer conjugate tags enable multiplexed assay in single cell format to distinguish a rare (for example a diseased) cell in a complex sample (for example, blood). The method can be used to identify leukemia cells in a patient's blood sample by employing metal-polymer tags conjugated to specific antibodies that recognize cell surface antigens present on the leukemia cells. For example, a positive multiplex staining of some cells in the peripheral blood mononuclear sample with antibodies against CD33, CD34, CD38, CD13, CD15, CD36 (tagged with different metals) and analyzed in a mass spectrometer based flow cytometer will indicate that the patient is developing acute monoblastic leukemia (AML-M5). In a similar manner, this method can be used to identify and quantify other cells, or particles.

Example 9 Kits

The invention encompasses kits useful for the preparation for the element tags and for carrying out the methods of the invention. The kits can include at least one of the following items:

a polymer comprising at least one metal-binding pendant group which contains at least one metal atom or is capable of binding at least one metal atom and further comprising a functional group that allows the polymer to be attached to one of a linker, a spacer, or a biomolecule, a metal solution, reagents for the attachment of the linker, spacer or biomolecule to the polymer, reagents for attachment of a functional group to the linker or the spacer, reagents for attachment of a metal to the polymer, affinity reagents including antibodies, buffers, instructions for preparing the element tag, instructions for attaching the element tag to an affinity reagent, instructions for attaching a metal to the element tag. and instructions for using the element tags for the analysis of analytes by elemental analysis. For example, the polymer can be homopolymers or copolymers of acrylamides, methacrylamides, acrylate esters, methacrylate esters, acrylic acid and methacrylic acid. The reagents can be chosen from at least one of the following: TCEP (tri(2-carboxyethyl) phosphine), Ligand-Polymer-Linker-Spacer Conjugate, phosphate buffer, TBS (tris-buffered saline), EDTA (Diaminoethanetetraacetic acid), ammonium acetate buffer, antibodies, metal salt solution, lanthanide salt solution, blocker buffers, washing buffers, FBS (fetal bovine serum), DMEM (Dulbecco's Modified Eagle's Medium), BSA (bovine serum albumin), dithiothreitol, bismaleimide, and DMF (dimethylformamide). The polymer can be provided which is attached to a linker or attached to both a linker and a spacer.

All references cited are incorporated by reference.

While the applicant's teachings are described in conjunction with various embodiments, it is not intended that the applicant's teachings be limited to such embodiments. On the contrary, the applicant's teachings encompass various alternatives, modifications, and equivalents, as will be appreciated by those of skill in the art.

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What is claimed is:

1. A method for the analysis of an analyte in a sample, comprising:

(i) incubating an element tagged affinity reagent with an analyte, the element tagged affinity reagent comprising an affinity reagent tagged with an element tag, the element tag comprising a linear or branched polymer having multiple metal-binding pendant groups, wherein each pendant group includes at least one metal atom or is capable of binding at least one metal atom, and wherein the affinity reagent specifically binds with the analyte, wherein the analyte is located within or on an intact cell;

(ii) separating unbound element tagged affinity reagent from bound element tagged affinity reagent; and

(iii) analyzing the element tag bound to the affinity reagent attached to the analyte of the intact cell by atomic spectroscopy, wherein analyzing occurs without prior acidification of the sample.

2. The method of claim **1**, wherein incubating the element tagged affinity reagent with the analyte comprises:

incubating two or more differential element tagged affinity reagents with two or more analytes, wherein the element tagged affinity reagents specifically bind with the two or more analytes to produce two or more differentially tagged analytes, wherein analyzing the element tag bound to the affinity reagent comprises analyzing the differential element tags bound to the two or more analytes by atomic spectroscopy.

3. The method of claim **1**, wherein the affinity reagent is further labeled with a fluorescent label.

4. The method of claim **1**, wherein the cell is a diseased cell.

5. The method of claim **4**, wherein the diseased cells is a leukemia cell or a progenitor of a leukemia cell.

6. The method of claim 1, wherein the elemental analysis is ICP-MS.

7. The method of claim 1, wherein the elemental analysis is by a mass spectrometer based flow cytometer.

8. A method for the analysis of an analyte of a sample, comprising:

(i) incubating element tags with an analyte so that the element tags bind the analyte, each element tag comprising a linear or branched polymer having multiple metal-binding pendant groups, wherein each pendant group includes at least one metal atom or is capable of binding at least one metal atom, wherein the element tags specifically bind the analyte, and wherein the analyte is located within or on an intact cell;

(ii) separating unbound element tags from bound element tags; and

(iii) analyzing the bound element tags of an intact cell by atomic spectroscopy, wherein analyzing occurs without prior acidification of the sample.

9. The method of claim 8, wherein incubating the element tags with the analyte comprises:

incubating two or more differential element tags with two or more analytes, to produce two or more differentially tagged analytes, and wherein analyzing the bound element tags comprises analyzing the differential tags bound to the two or more analytes by atomic spectroscopy.

10. The method of claim 1, further comprising obtaining fluorescence from the intact cell.

11. The method of claim 10, wherein the affinity reagent is further labeled with a fluorescent label.

12. The method of claim 10, wherein the obtained fluorescence is obtained by fluorescent microscopy.

13. The method of claim 10, further comprising presorting the intact cell, prior to elemental analysis, based on the obtained fluorescence.

14. The method of claim 1, wherein the affinity reagent is an antibody.

15. The method of claim 1, wherein the affinity reagent specifically binds biotin.

16. The method of claim 8, further comprising obtaining fluorescence from the intact cell.

17. The method of claim 16, wherein at least one of the element tags includes a fluorescent label.

18. The method of claim 16, wherein the obtained fluorescence is obtained by fluorescent microscopy.

19. The method of claim 16, further comprising presorting the intact cell, prior to elemental analysis, based on the obtained fluorescence.

* * * * *

EXHIBIT C

US010436698B2

(12) **United States Patent**
Bandura et al.

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(45) **Date of Patent: *Oct. 8, 2019**

(54) **MASS SPECTROMETRY BASED MULTI-PARAMETRIC PARTICLE ANALYZER**

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This patent is subject to a terminal disclaimer.

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(Continued)

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CPC ***G01N 15/1404*** (2013.01); ***H01J 49/004*** (2013.01); ***H01J 49/04*** (2013.01);
(Continued)

(58) **Field of Classification Search**
None
See application file for complete search history.

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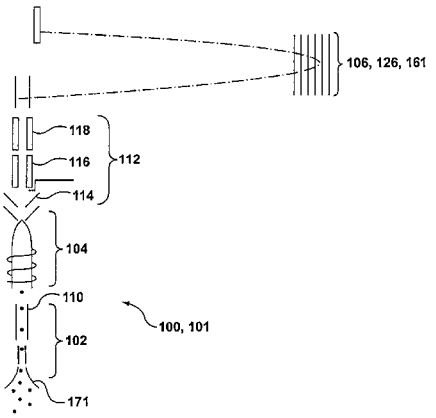
(57) **ABSTRACT**

An analytical instrument has a sample introduction system for generating a stream of particles from a sample. An ionization system atomizes and ionizes particles in the stream as they are received. The instrument has an ion pretreatment system and a mass analyzer. The ion pretreatment system is adapted to transport ions generated by the ionization system to the mass analyzer. The mass analyzer is adapted measure the amount of at least one element in individual particles from the stream by performing mass analysis on the ions from the atomized particles. The instrument can be adapted to measure the amount of many different tags, for example at least five different tags, at the same time to facilitate multi-parametric analysis of cells and other particles.

6 Claims, 11 Drawing Sheets



Related U.S. Application Data

continuation of application No. 13/294,799, filed on Nov. 11, 2011, now Pat. No. 9,952,134, which is a continuation of application No. 12/332,812, filed on Dec. 11, 2008, now abandoned, which is a division of application No. 11/089,023, filed on Mar. 25, 2005, now Pat. No. 7,479,630.

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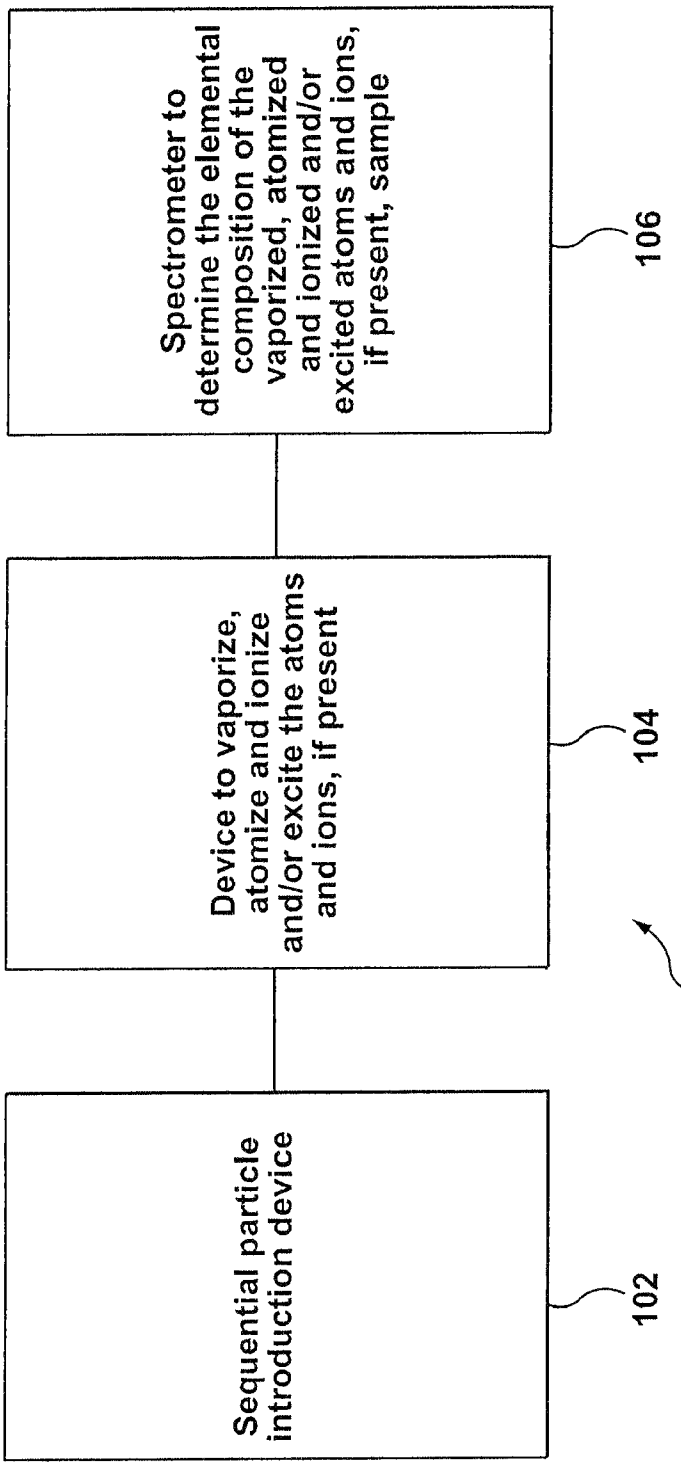

Spectrometer to determine the elemental composition of the vaporized, atomized and ionized and/or excited atoms and ions, if present, sample
106
Device to vaporize, atomize and ionize and/or excite the atoms and ions, if present
104
100
Sequential particle introduction device
102

FIG. 1

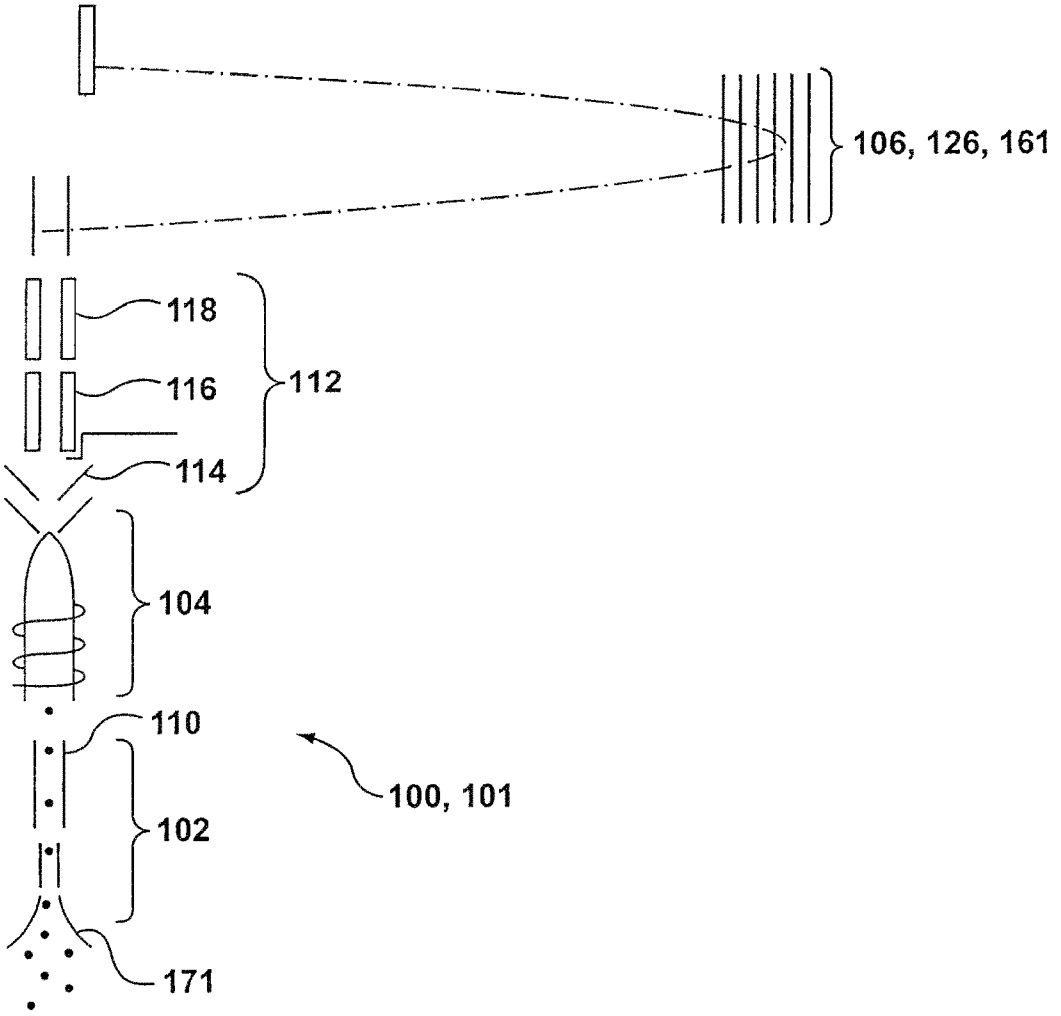


FIG. 2

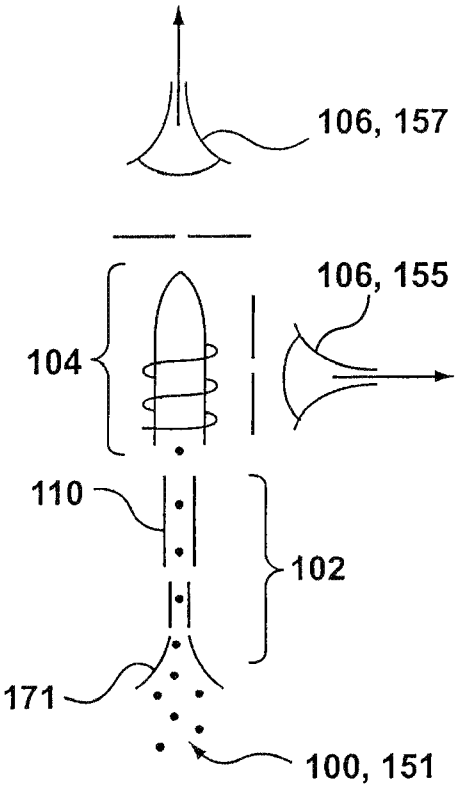


FIG. 3

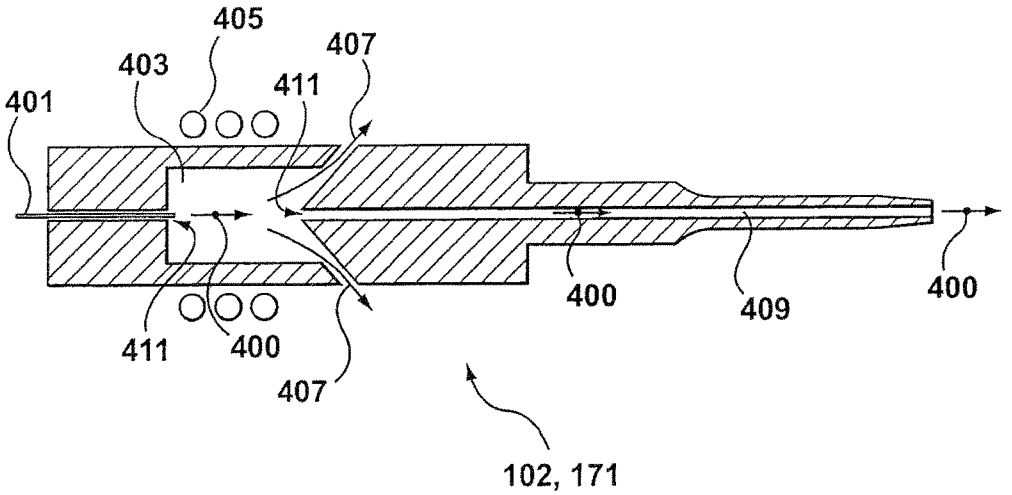

405
403
407
411
401
411
400
407
400
409
400
102, 171

FIG. 4

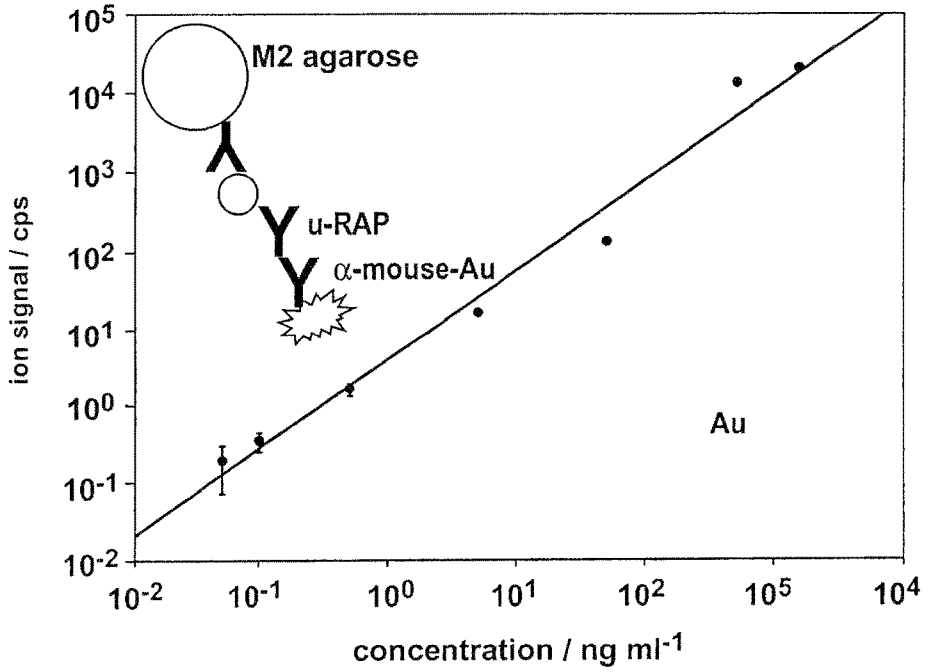

M2 agarose
u-RAP
α-mouse-Au
Au
ion signal / cps
concentration / ng ml-1
10^5
10^4
10^3
10^2
10^1
10^0
10^-1
10^-2
10^-2
10^-1
10^0
10^1
10^2
10^5
10^4

FIG. 5

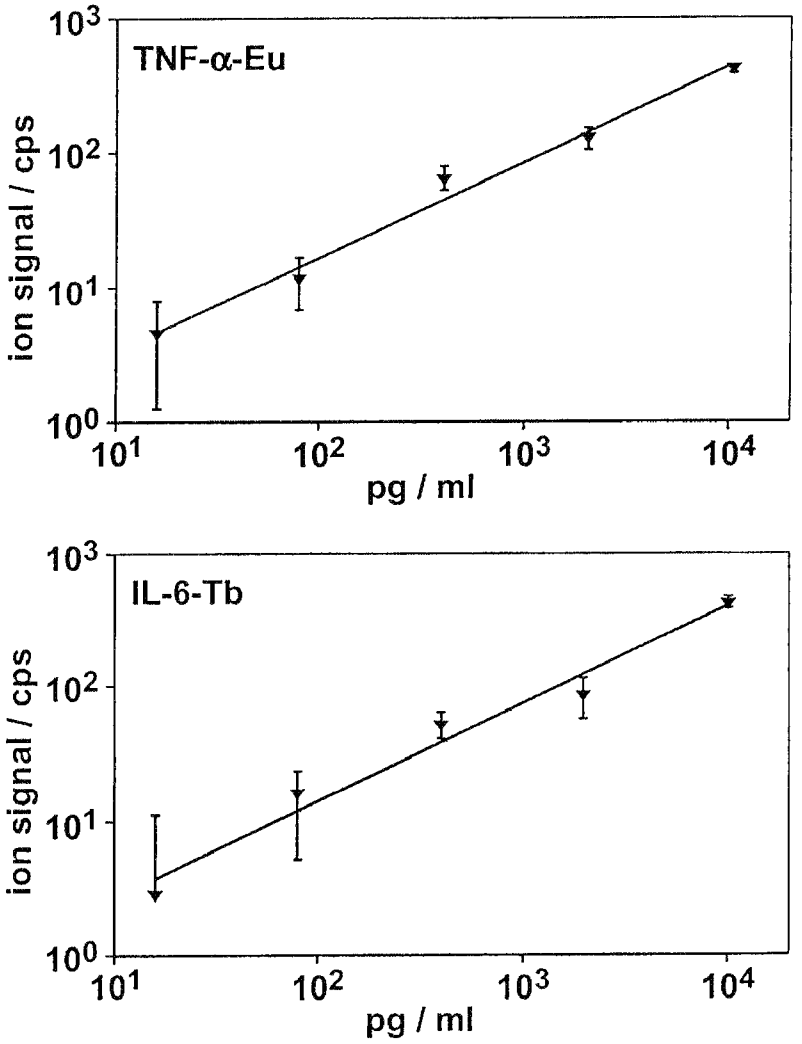


FIG. 6

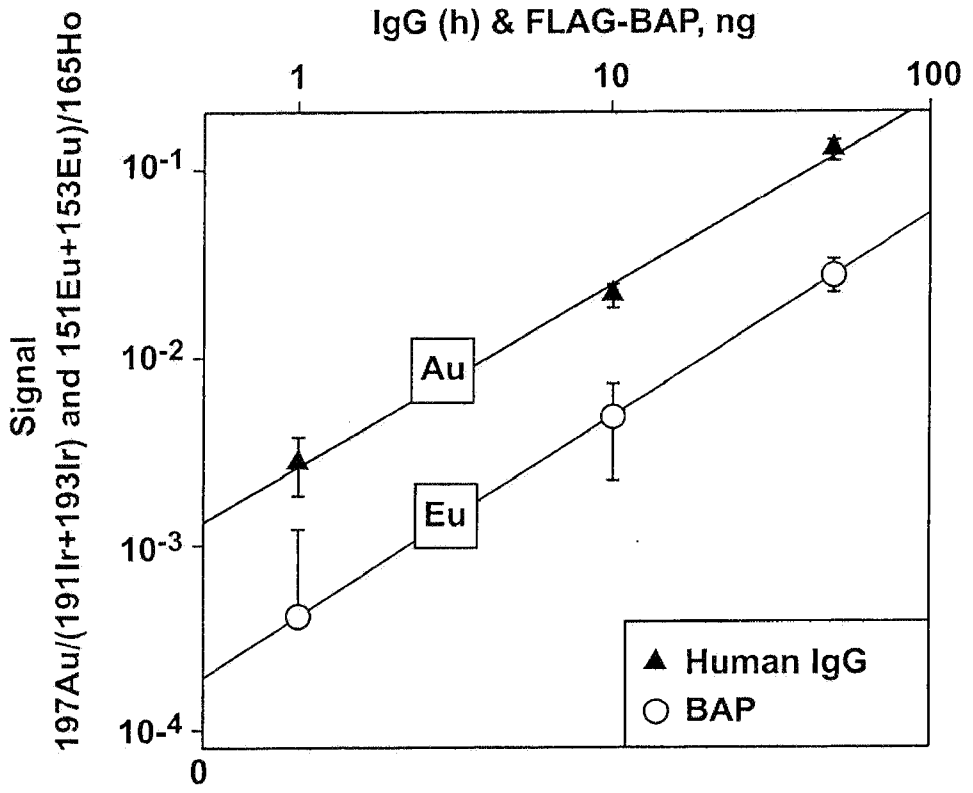

IgG (h) & FLAG-BAP, ng
1
10
100
Signal
197Au/(191Ir+193Ir) and 151Eu+153Eu)/165Ho
10-1
10-2
10-3
10-4
0
Au
Eu
Human IgG
BAP

FIG. 7

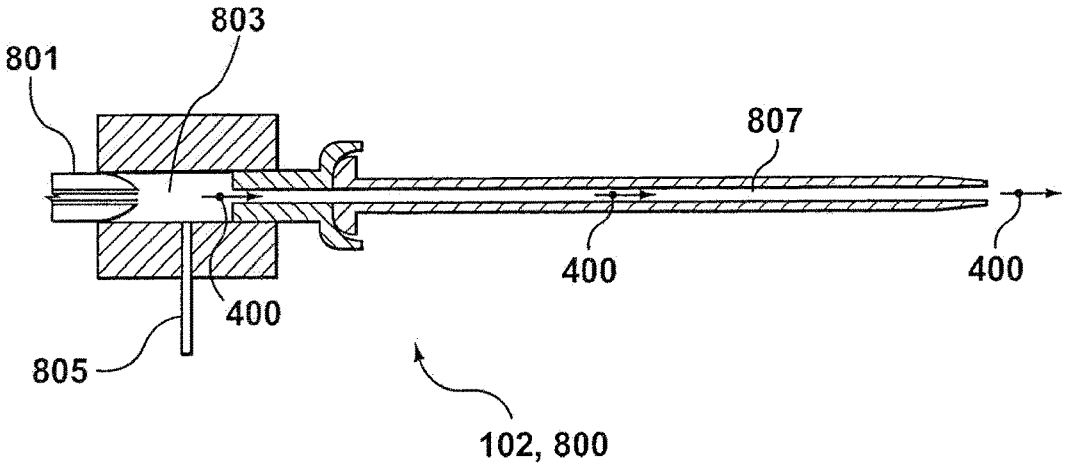

803
801
807
400
400
400
805
102, 800

FIG. 8

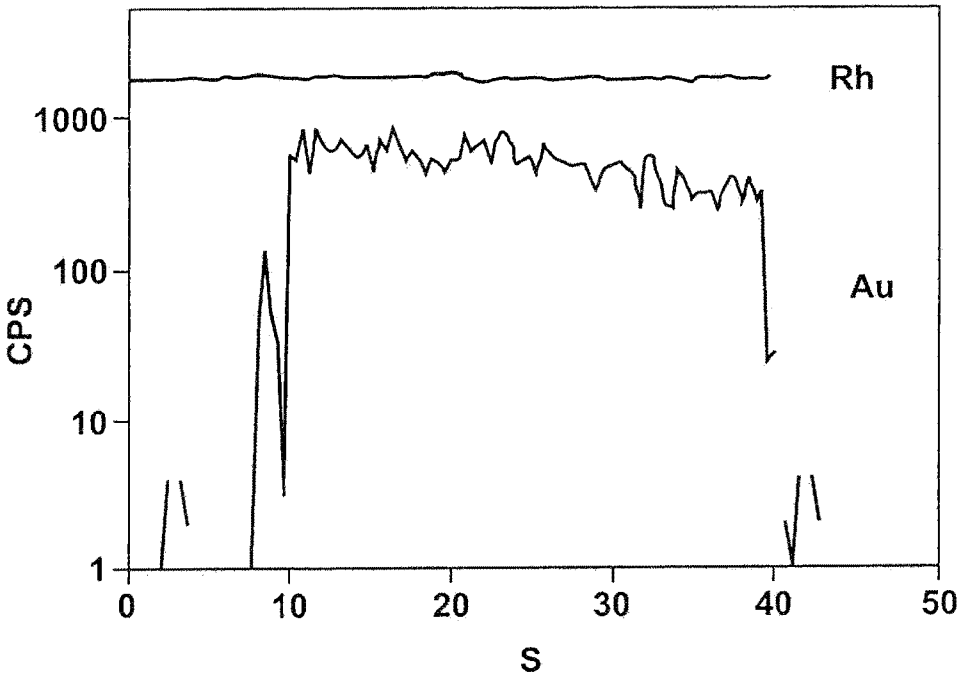
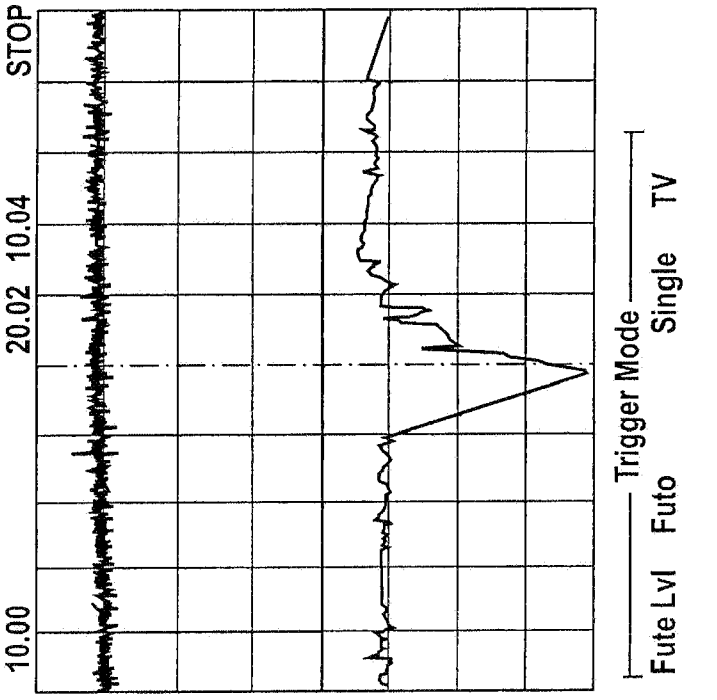
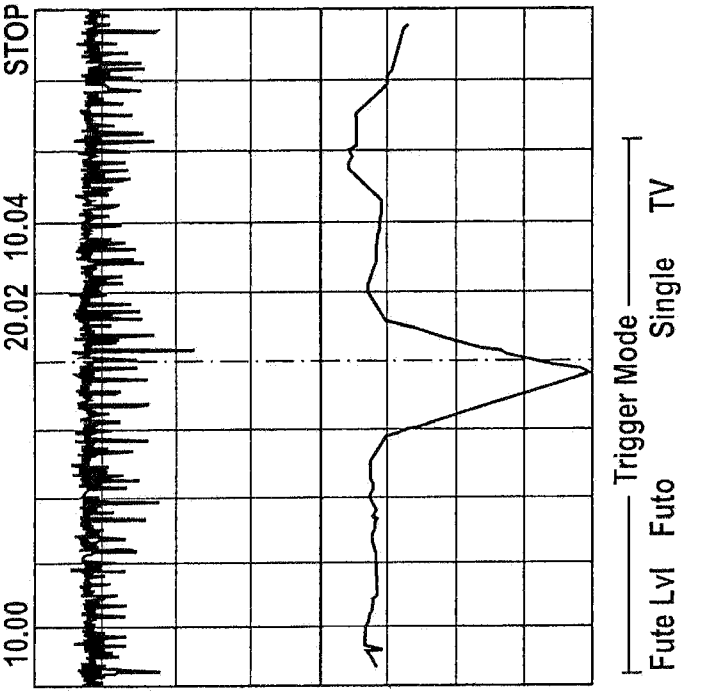

Rh
Au
1000
100
10
1
CPS
0
10
20
30
40
50
S

FIG. 9



Au^+ from cells: single ion (lower trace, 20 ns/div) and no multiple ions (upper trace, 10 us/div):
10 mV/div

FIG. 10B



Ar_2^+: single ion (lower trace, 20 ns/div) and multiple ions (upper trace, 10 us/div):
40 mV/div

FIG. 10A

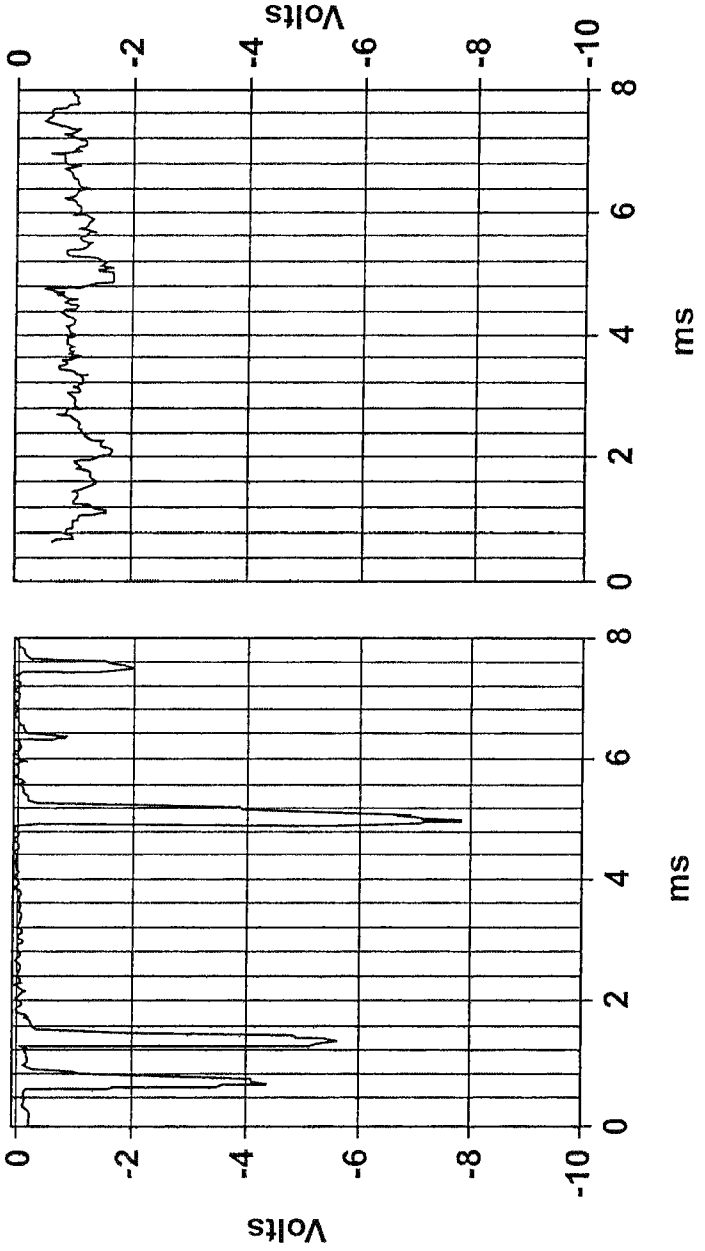


FIG. 11B

FIG. 11A

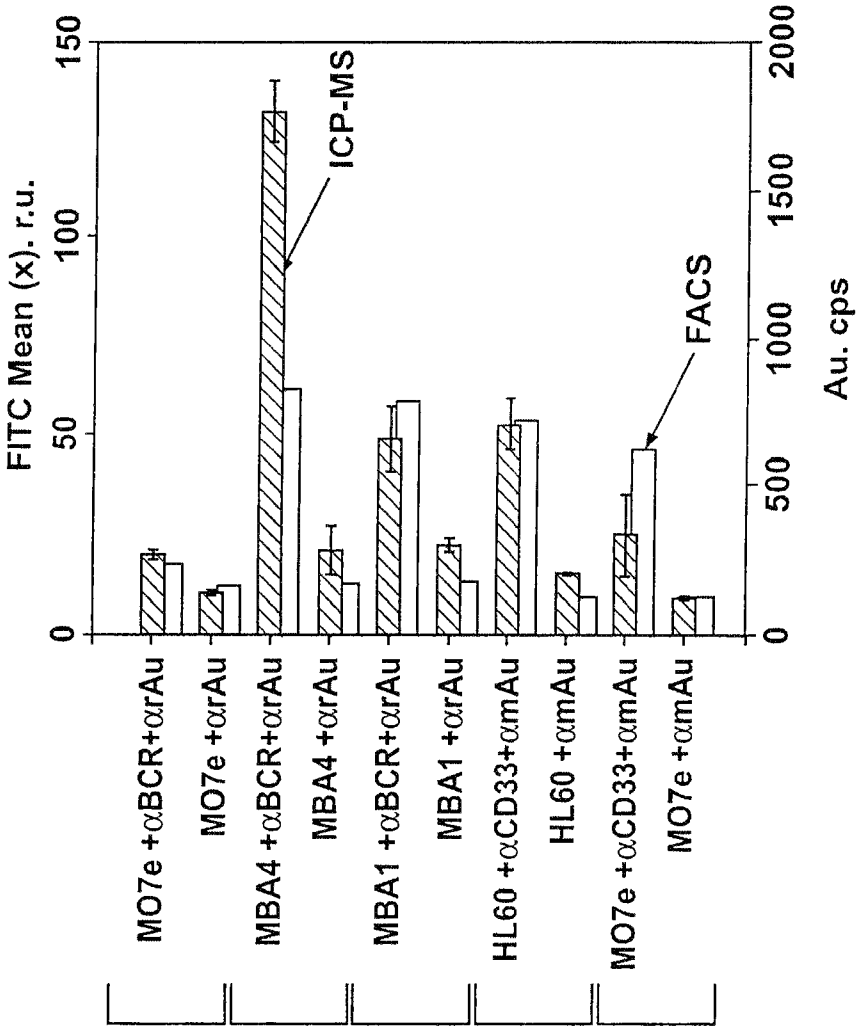

FITC Mean (x). r.u.
0
50
100
150
Au. cps
0
500
1000
1500
2000
ICP-MS
FACS
MO7e +αBCR+αrAu
MO7e +αrAu
MBA4 +αBCR+αrAu
MBA4 +αrAu
MBA1 +αBCR+αrAu
MBA1 +αrAu
HL60 +αCD33+αmAu
HL60 +αmAu
MO7e +αCD33+αmAu
MO7e +αmAu

FIG. 12

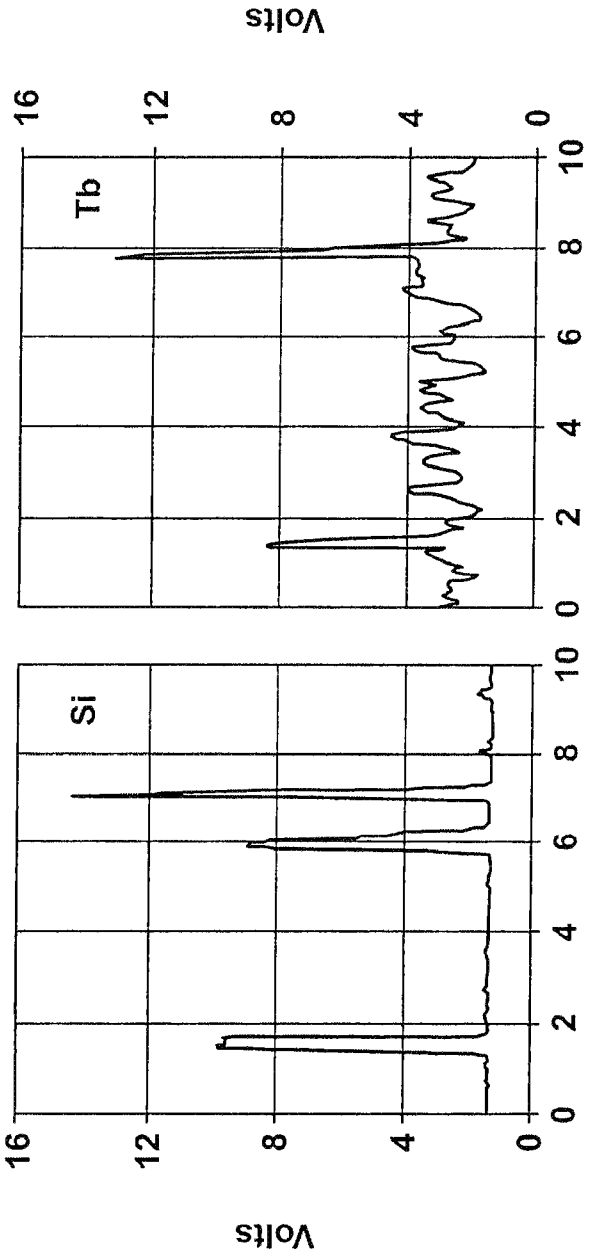


FIG. 13B

FIG. 13A

MASS SPECTROMETRY BASED MULTI-PARAMETRIC PARTICLE ANALYZER

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 15/888,871, filed Feb. 5, 2018, which is a continuation of U.S. patent application Ser. No. 13/294,799, filed Nov. 11, 2011, which is a continuation of U.S. patent application Ser. No. 12/322,812, filed Dec. 11, 2008, which is a divisional of U.S. Pat. No. 7,479,630, issued Jan. 20, 2009, which is a non-provisional of U.S. Patent Application Ser. No. 60/555,952, filed Mar. 25, 2004, all of which are hereby incorporated by reference.

The entire contents of U.S. patent application Ser. No. 09/905,907, filed Jul. 17, 2001 and entitled Elemental Analysis of Tagged Biologically Active Materials (published as US 2002/0086441); and Ser. No. 10/614,115, filed Jul. 3, 2003 and entitled Elemental Analysis of Tagged Biologically Active Materials (published as US 2004/0072250) are hereby incorporated by reference.

The entire contents of U.S. Pat. No. 6,524,793, filed Jun. 18, 1999 and entitled Multiplexed Analysis of Clinical Specimens Apparatus and Method; International Patent Application Publication WO 98/33203, published Jul. 30, 1998, and entitled Gate for Eliminating Charged Particles in Time of Flight Spectrometers; and each of the publications cited in the Reference Section herein are hereby incorporated by reference.

FIELD OF THE INVENTION

The invention features apparatus and methods for sequentially analyzing particles, for example single cells or single beads, by spectrometry. In particular, the invention provides elemental-flow cytometers.

BACKGROUND OF THE INVENTION

The ability to analyze single particles, for example single cells or single beads, is a useful tool in the health sciences, in human and animal food sciences, in environmental sciences, forensic sciences, and in genomics and proteomics.

In the health sciences, cells are recognized as members of certain classes, for example normal cells or cancerous cells for diagnostic or biomedical research purposes. Cells carry multiple antigens or biomarkers [1], either extracellularly or intracellularly [2], which can be quantified or qualified for clinical medicine [3] or biomedical research [4] purposes. These methods are useful for development of pharmaceutical products particularly in the development of cell based assays and toxicity studies.

For example, chronic lymphocytic leukemia (CCL) is recognized as a unique disorder of B-cells [5, 6]. CCL is a disease with an uncertain clinical picture, and is often misdiagnosed resulting in inadequate treatment. However, a more detailed study of a patient's cellular immunophenotypic profile allows reclassification of the patient, which leads to a more personalized diagnosis and treatment. Such classification requires multi-targeted analysis of many markers on a cell membrane as well as in-cell antigens, their qualitative and quantitative description, and consideration of minute concentration variances.

Other examples in the health sciences include the analysis of single cells in the subclassification of non-Hodgkin's lymphoma. In addition, single cell analysis is useful in immunophenotyping of helper T-cells, and the determination of the ratio of CD4 to CD8 T-cells, for indication of the HIV progression in HIV positive patients. Further, the technique can be used to analyze single cells from patients with renal, cardiac and bone marrow transplants, for discriminating between graft rejections and viral infections in post-operative patients.

In human and animal food sciences, the analysis of single cells can be used to detect artificial hormones, pesticides, herbicides or antibiotics. Finally, in environmental sciences, the analysis of single cells can detect toxic waste, for example, in plant or bacterial cells.

A known method of analyzing single cells is by a fluorescence activated cell sorter (FACS). FACS is a technology to measure biological properties of cells by scanning single cells as they pass through a laser beam. Cells are usually stained with one or more fluorescent dyes specific to cell components of interest, for example, receptors on the cell surface and DNA of the cell nucleus, and the fluorescence of each cell is measured as it traverses the excitation beam. Since the amount of fluorescence emitted is proportional to the amount of fluorescent probe bound to the cell antigen, antibodies conjugated to fluorochromes are routinely used as reagents to measure the antigen both qualitatively and quantitatively on and in the cell. Primarily, researchers use the sorting function of the FACS machines to investigate cell receptors and other membrane antigens on a specific cell population. It can be used for antibody screening in multiple cell lines simultaneously (for example, a transfected cell line expressing the antigen of interest and a control cell line not expressing the antigen). In its simplified flow cytometry function, FACS machines are used mostly without sorting, which allows for example the use of fixed permeabilized cells and analysis of intracellular antigens. Many routine flow cytometry methods that identify antigens expressed on the cell surface and within the cell using specific antibodies, as well as general immunoassay methods for clinical diagnostics and treatment have been developed. Some of them involve multiplexing through the use of different fluorochromes and lasers. Deficiencies of this approach are related to limitations and difficulties of cell staining methods and spectral overlap of fluorochromes. Other measurable optical parameters include light absorption and light scattering, the latter being applicable to the measurement of cell size, shape, density, granularity, and stain uptake.

U.S. patent application Ser. No. 09/905,907, published under US 2002/0086441 on Jul. 4, 2002, and Ser. No. 10/614,115, describe labeling of analytes for analysis by mass spectrometry. Biologically active materials (for example, antibodies and aptamers) are labeled and conjugated to analytes prior to analysis.

SUMMARY OF THE INVENTION

In one broad aspect, the present invention provides an apparatus for introducing particles sequentially and analyzing the particles (for example, single particles such as single cells or single beads), by spectrometry. The apparatus, an elemental flow cytometer, is an instrument comprising: a means for introducing single particles sequentially, a means to vaporize, atomize, and excite or ionize the particles or an elemental tag associated with an analyte on the particles, and a means to analyze the elemental composition of the vaporized, atomized, ionized and/or excited particles, or an elemental tag associated with the particles.

It is to be understood that although the term "means for introducing single particles sequentially" is used, this may encompass introduction of a predetermined number of particles (for example, 2 or more) in discrete 'packets'.

It is also to be understood that the term "means to vaporize, atomize, and excite or ionize" includes means where atomization may not be necessary, so that the term may or may not encompass vaporization followed by ionization directly. In some applications, such as for example optical emission spectrometry (OES), it is not essential to ionize the sample; emission from atomic species can be sufficient. For OES, it is necessary only to excite the atoms (or ions) to cause emission. Thus, for example, "vaporize, atomize and ionize" should be understood to mean vaporize, atomize and ionize (for mass spectrometry) or excite (either or both atoms and ions) for OES.

Another aspect of the invention is an analytical instrument. The instrument has a sample introduction system for generating a stream of particles from a sample. An ionization system receives particles in the stream. The ionization system is operable to atomize particles in the stream as the particles are received from the sample introduction system and to ionize atoms from the atomized particles. The instrument has an ion pretreatment system and a mass analyzer. The ion pretreatment system is adapted to transport ions generated by the ionization system to the mass analyzer. The mass analyzer is adapted measure the amount of at least one element in individual particles from the stream by performing mass analysis on the ions from the atomized particles.

Another aspect of the invention is an instrument for performing multi-parametric quantitative analysis of particles in a stream of particles. The instrument has a sample introduction system for generating a stream of particles from a sample. A particle analyzer is adapted to measure the amount of each of a plurality of at least five different tags in each of a plurality of particles in the stream of particles produced by the sample introduction system. The particle analyzer has a detector adapted to generate signals corresponding to each tag. The signals generated by the detector corresponding to each of the tags is independent from the signal generated by the detector corresponding to the others of the tags.

In another broad aspect, the invention provides a method for analyzing particles that have been introduced sequentially, such as single cells or single beads, by spectrometry. A trigger will report the ion cloud arrival with following analysis, including for example initiation of data acquisition. Triggering may be based, for example on light scattering or on an ion current change or ion composition change.

Another aspect of the invention is an elemental flow cytometer, comprising: a means for introducing particles sequentially into a device to vaporize, atomize and excite or ionize the particles, or an elemental tag associated with the particles; a device to vaporize, atomize and excite or ionize the particles, or an elemental tag associated with the particles, downstream of the means for introducing particles sequentially; and a spectrometer to analyze the vaporized, atomized and ionized and/or excited particles, or the elemental tag associated with the particles.

Another aspect of the invention is a mass-spectrometer-based flow cytometer, comprising: a means for introducing particles sequentially into a device to vaporize, atomize and ionize the particles, or an elemental tag associated with the particles; a device to vaporize, atomize and ionize the particles, or an elemental tag associated with the particles, downstream of the means for introducing particles, sequentially; and a mass spectrometer operatively connected and downstream of the device to vaporize, atomize and ionize.

Another aspect of the invention is a mass-spectrometer-based flow cytometer, comprising: a means for introducing particles sequentially into a device to vaporize, atomize and ionize the particles, or an elemental tag associated with the particles; a device to vaporize, atomize and ionize the particles, or an elemental tag associated with the particles, downstream of the means for introducing particles sequentially; an ion pretreatment device operatively connected and downstream of the device to vaporize, atomize and ionize; and a mass spectrometer operatively connected and downstream of the ion pretreatment device. The ion pretreatment device may be provided as a part of the mass spectrometer, preferably upstream of the mass analyzer section thereof.

Another aspect of the invention, is an optical emission spectrometer-based flow cytometer, comprising: a means for introducing particles sequentially into a device to vaporize, atomize and excite or ionize the particles, or an elemental tag associated with the particles; a device to vaporize, atomize and excite or ionize the particles, or an elemental tag associated with the particles downstream of the means for introducing particles sequentially, and an optical emission spectrometer to analyze the vaporized, atomized and excited or ionized particles, or the elemental tag associated with the particles downstream of the device to vaporize, atomize and excite or ionize the particles.

Another aspect of the invention, is a method of analyzing particles that have been introduced sequentially into a device to vaporize, atomize and excite or ionize, comprising: sequentially introducing particles or particles associated with an elemental tag, into a device to vaporize, atomize and excite or ionize the particles or the elemental tag associated with the particles; and introducing the vaporized, atomized and excited or ionized particles, or the elemental tag associated with the particles into a spectrometer.

The labeling or tagging of the single particles with elemental tags can be done, for example, using the methods and system disclosed in U.S. Ser. No. 09/905,907 and U.S. Ser. No. 10/614,115, both applications of which are herein incorporated by reference. U.S. Ser. No. 09/905,907 and U.S. Ser. No. 10/614,115 describe methods and systems for the analysis of biologically active materials conjugated to analytes by mass spectrometry. Other methods of labeling or tagging the particles will also serve. If, for example, the particles are beads, the particles themselves can be labeled either on the surface or within their bodies, as disclosed herein.

Another aspect of the present invention is to provide kits having reagents for carrying out the methods of the present invention and instructions for these methods.

Another aspect of the present invention is to provide beads with an affinity substance as a carrier to measure an analyte in a sample, further comprising an elemental label or tag. The elemental tag can be on the analyte, on the affinity substance or (and) on or in the bead itself.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a schematic diagram of a flow cytometer according to the invention.

FIG. **2** is a schematic diagram of an embodiment of a mass-spectrometer-based flow cytometer according to the invention.

FIG. **3** is a schematic diagram of an embodiment of an optical emission spectrometer (OES)-based flow cytometer of the invention.

FIG. **4** is a schematic diagram of a single-particle injector according to the invention.

FIG. **5** is a calibration curve for flag-BAP using agarose bead immobilization with α-BAP primary and Au-tagged α-mouse secondary antibodies.

FIG. **6** is a plot of Fluorokine bead assay, detecting TNF-.alpha. and IL-6 simultaneously using distinguishable (Eu and Tb) elemental tags on the corresponding primary antibodies.

FIG. **7** is a plot of an ELISA based assay coupled to ICP MS showing the simultaneous quantitation of two proteins.

FIG. **8** shows a schematic diagram of a sample introduction system

FIG. **9** shows overlaid results of measuring ion signals as a function of time for direct injection of a standard solution of 100 ppt Rh (1% HNO_3) and of a MOTE cell suspension for which the surface antigen CD33 was tagged with a Au particle.

FIG. **10**A shows oscilloscope output of an Ar_2^+ signal from MO7e cell introduction for which the surface antigen CD33 was tagged with a Au particle.

FIG. **10**B shows oscilloscope output of an Au^+ signal from MO7e cell introduction for which the surface antigen CD33 was tagged with a Au particle.

FIG. **11**A shows an analog signal from an oscilloscope registered while continuously monitoring Na^+. in a cell suspension in a 30 mM $CaCl_2$ buffer.

FIG. **11**B shows an analog signal from an oscilloscope registered while continuously monitoring Na^+. for a 30 mM $CaCl_2$ buffer.

FIG. **12** shows comparative data for analysis of cell surface proteins and intracellular proteins by both conventional FACS and by the method of the present invention.

FIG. **13**A shows Si^+ signal for stober silica particles grown in the presence of a Tb solution.

FIG. **13**B shows Tb^+ signal for stober silica particles grown in the presence of a Tb solution.

DEFINITIONS

ICP-MS: is an Inductively-Coupled Plasma Mass Spectrometer.

FACS: is a Fluorescence Activated Cell Sorter.

Various aspects of the present disclosure are described herein with reference to single particles. However, in some cases, these aspects of the present disclosure can be used with packets of a predetermined number of discrete entities (e.g., 2, 3, or 4). Various aspects of the present disclosure as described herein can be used with single cells, single beads, single bacteria, single viral particles, single pollen particles, single microscopic insects such as dust mites.

Tag (or label): a chemical moiety that provides a distinguishable signal of the presence of the analyte or analyte complex with which it is associated, as for example through linkage to an affinity product that in tum recognizes the analyte or analyte complex. As disclosed herein, the tag (which is also called an "elemental tag") can contain an element or an isotope (or multiple copies thereof) that provide the distinguishable signal. A tag can include for example an element or isotope of an element that is associated with an analyte or analyte complex and which is measured to determine the presence of the analyte. A tag can also include, for example, any distinguishable component (e.g., an element or isotope or multiple copies thereof) that is provided on the surface or within the body of, or is otherwise associated with, a particle and serves to distinguish that particle from other particles.

TOF-MS: is a Time-of-Flight Mass Spectrometer

DESCRIPTION OF SPECIFIC EMBODIMENTS INCLUDING THE BEST MODE CURRENTLY CONTEMPLATED BY THE INVENTORS

The elemental flow cytometer of the present invention provides for the identification and quantitative analysis of particles that have been introduced sequentially into a device to vaporize, atomize and excite or ionize them, for example individual cells or microscopic beads, by measuring the elemental composition of the single particle (or any distinctive part of cell or bead), or a tag or label associated with an analyte located on or in the cell or bead by employing the mass-to-charge ratio or optical emission of the disintegrated tag elements. The tag can be of any chemical nature, as it is only its elemental composition that is important. In comparison, the chemical structure of the appropriate tag is absolutely critical to provide a unique fluorescence in FACS.

The elemental flow cytometer includes:

means for introducing particles sequentially (for example, cell-by-cell or bead-by-bead), preferably adapted for discrete event analysis;

means to vaporize, atomize and excite or ionize the particles,

or an elemental tag (or classifiable elemental composition) associated with an analyte of interest on or in the particles to quantify the analyte of interest associated with the particles;

and means for registering the information on elemental composition of the particles, or an elemental tag associated with an analyte on the particles. This can be done, for example, by mass spectrometry (MS) or by optical emission spectrometry (OES).

Elemental flow cytometers according to the invention are quantitative analytical instruments [7]. They can perform the task of quantitative or qualitative analysis of biological or environmental samples using analytical methods [8].

Beads with an affinity substance can be used as carriers to measure an analyte in a sample. The placement of the elemental tag or label can be on the analyte, on the affinity substrate, and/or on or in the bead itself.

Specific embodiments of the elemental flow cytometer include: (1) a mass spectrometer based flow cytometer (MS FC) and (2) an optical emission spectrometer based flow cytometer (OES FC).

A mass spectrometer based flow cytometer (MS FC) comprises:

means for introducing particles sequentially:

means to vaporize, atomize and ionize the particles and/or any tags that may be associated with the particles; and

a mass spectrometer to analyze the elemental composition of the vaporized, atomized and ionized particles, and/or any tags that may be associated with the particles.

MS FCs according to the invention can further comprise ion pretreatment devices, for pretreatment of ions prior to analysis by the mass spectrometer.

The means to vaporize, atomize and ionize the single particles may include glow discharge, graphite furnace, and capacitively coupled plasma devices, or other suitable devices. Preferably, the means to vaporize, atomize and ionize the single particle includes an inductively coupled plasma (ICP) device because it has a capacity to disintegrate, vaporize, atomize and ionize cells and beads during their short residence time in the plasma and because the ICP is particularly tolerant of concomitant materials, is robust to

changes of the composition of the plasma gases, and is a highly efficient atomizer and ionizer.

The ion pretreatment device acts, inter alia, as an interface between atmospheric conditions in the vaporizer/atomizer/ionizer and the vacuum in the mass spectrometer. In addition, the very strong ion current originating from this source is dominated by space charge, which could be reduced by an accelerating potential and/or by rejection of major plasma ions on the basis of their mass-to-charge ratio (Ar^+, for example). In the case of a TOF MS, the ion pretreatment device also conditions the ion flow for the needs of the TOF mass analyzer. For example, it will narrow the ion energy distribution and focus the parallel ion beam close to the axis of the mass analyzer.

The mass spectrometer can be any mass spectrometer. For example, it can be a quadrupole, magnetic sector with array detector, 3D Ion Trap or Linear Ion Trap mass spectrometer. Preferably it is a time of flight mass spectrometer (TOF MS). TOF MS is a simultaneous analyzer. It is able to register all masses of interest in one particle simultaneously.

The optical emission spectrometer based flow cytometer (OES FC) comprises:

a means for introducing particles sequentially; a means to vaporize, atomize and excite or ionize the particles, and/or any tags that may be associated with the particles; and

an optical emission spectrometer to analyze the elemental composition of the vaporized/atomized and excited or ionized particles and/or any tags that may be associated with the particles.

The means to vaporize, atomize and excite or ionize the single particles may include glow discharge, graphite furnace, and capacitively coupled plasma devices, or other suitable devices. Preferably, the means to atomize and ionize the single particles includes an inductively coupled plasma (ICP) device because it has a capacity to disintegrate, atomize and excite or ionize cells and beads during their short residence time in the plasma and because the ICP is particularly tolerant of concomitant materials, is robust to changes of the composition of the plasma gases, and is a highly efficient atomizer and ionizer.

Processes implemented by elemental flow cytometers according to the invention can also include an in-line lysis step between the means for single particle introduction and the means to vaporize, atomize and ionize.

The embodiments will now be described in detail.

In a most general aspect, the present invention provides an elemental analyzer as a detector for a flow cytometer. FIG. **1** shows schematically a cytometer **100** suitable for use implementing methods of analysis according to the invention. Cytometer **100** comprises means **102** for introducing particles sequentially, for example a cell or particle injector **171** (FIGS. **2**, **3**, **4**), operatively connected upstream of a device **104** for vaporizing, atomizing and exciting or ionizing particles or elemental tags associated with the particles. The elemental composition of the particle or elemental tag is determined by a spectrometer **106** operatively connected to the device **104**. Spectrometer **106** may, for example, include an optical spectrometer **157**, which detects the emission from excited atoms and/or ions, or a mass spectrometer **116** which detects the ions.

In one embodiment the present invention provides a mass-spectrometer based flow cytometer (MS FC) **101**. A schematic representation of such an embodiment is given in FIG. **2**.

Referring to FIG. **2**, mass-spectrometer based embodiment **101** of cytometer **100** comprises means **102** for introducing particles sequentially, for example a cell or particle injector **171**, operatively connected upstream of device **104** for vaporizing, atomizing and exciting or ionizing particles or elemental tags associated with the particles, namely an inductively coupled plasma (ICP) vaporizer/atomizer/ionizer. In the embodiment shown, means **102** comprises optional in-line lysis system **110**.

Ion pretreatment device **112**, in this instance comprising vacuum interface **114**, high-pass filter **116** and gas-filled "cooler" cell **118**, is operatively connected downstream of the ICP vaporizer/ionizer.

Time-of-flight (TOF) mass spectrometer **106**, **161**, **126** is operatively connected downstream of the ion pretreatment device. Use of mass spectrometer-based cytometer **101** according to such embodiments to analyze single particles can provide greatly improved accuracy, large dynamic range and high sensitivity, compared to prior art systems. In addition, because a large number of distinguishable elements and isotopes can be used as tags, and because the mass spectrometer provides high abundance sensitivity (exceedingly small overlap of signal on adjacent mass/charge detection channels), it facilitates a higher order of multiplexing (simultaneous determination of multiple analytes, each distinguishably tagged) than prior art fluorescence-based detection flow cytometers. Further, because of the high resolution of adjacent mass/charge detection channels and the large linear dynamic range of the mass spectrometer, the instrument provides for a large dynamic range both for a given analyte and between analytes. Thus, in many instances generic tagging moieties can be used in analyses in which the copy-count of the analytes differs dramatically; this distinguishes the method from conventional fluorescence detection methods for which the composition of the several fluorophores used for multiplex assay must often be adjusted for a particular assay to provide emission intensities of similar magnitude to minimize spectral overlap. Thus such embodiments can provide researchers and clinicians substantially improved analytical and prognostic capabilities.

Another important application of cytometers according to this embodiment of the invention is to multiplex assay distinguishable beads, where the beads are distinguished by their elemental compositions and have attached affinity products that recognize an antigen in the sample into which they are introduced, where the antigen is then further recognized using a sandwich (or other) assay employing yet a further distinguishable element.

Significant components of the mass spectrometer-based flow cytometer **101** of FIG. **2** and methods of use will now be described in detail.

Tagging

In Certain Cases the Particle (For Example a Single Cell) Will Not Require Tagging

In some cases a particles will not require tagging. For example, if a single cell contains or is bound to an element that is detectable against the background by mass spectrometry, no tagging is required. For example, for the analyses of bacterial or plant cells that accumulate elemental species in bioremediation, additional tagging would not be required. Further, the intracellular accumulation of metal, for example platinum- or gold-containing drugs would not require additional tagging.

In Cases Where Single Particles Require Tagging

Tagging of particles can be done by many methods as is known to those of skill in the art. For example, fluorescent dyes which have a succinimidyl ester moiety react efficiently with primary amines of proteins (antibodies) to form stable dye-protein conjugates. In a first step to tag DNA, an amine-modified nucleotide, 5-(3-aminoallyl)-dUTP, can be

incorporated into DNA using conventional enzymatic tagging methods. In a second step, the amine-modified DNA can be chemically tagged using an amine-reactive fluorescent dye. Biotinilation of antibodies can be carried out using sulfhydryl-directed solid-phase chemistry. These methods are well established and are available in kit formats from different companies, including for example Molecular Probes Inc, Pierce Chemical Company, and others. Specific chemical reactions are known in radioimmunochemistry. For example, radionuclides (88/90)Y and (177)Lu can be used to tag antibodies using cyclic diethylenetriaminepentaacetic acid anhydride (cDTPA), isothiocyanatobenzyl-DTPA (SCN-Bz-DTPA), or 1,4,7,10-tetraazacyclododecanetetraacetic acid (DOTA)(PMID: 14960657).

Elemental analysis of tagged biologically active materials has been disclosed in the incorporated references, U.S. patent application Ser. Nos. 09/905,907 and 10/614,115. Tagged biologically active materials, for example, antibodies and aptamers, etc., that react specifically with cellular components can be used to tag cells. Other affinity products are known to those skilled in the art. For example, they may include antigens, RNA, DNA, lipoproteins, glycoproteins, peptides, polypeptides, hormones, etc.

Although in many applications of systems and methods according to the invention it is convenient to tag each biologically active material (for example an antibody, aptamer or antigen) with a single element or isotope, it should be readily appreciated by those skilled in the art that an antibody or antigen may be tagged with more than one element. As there are more than 80 naturally-occurring elements having more than 250 stable isotopes, there are numerous elements, isotopes, and combinations thereof to choose from. Within limits prescribed by the need to have distinguishable tags when in combination, this will allow for simultaneous detection of numerous biologically-tagged complexes. It is advantageous if the relative abundance of the tag elements is sufficiently different from the relative abundance of elements in a given sample under analysis. By "sufficiently different" it is meant that under the methods of the present invention it is possible to detect the target antibody or antigen over the background elements contained in a sample under analysis. Indeed, the difference in inter-elemental ratios of the tagged antibody or antigen, and the sample matrix can be used advantageously to analyze the sample.

It is feasible to select elemental tags, which do not produce interfering signals during analysis. Therefore, two or more analytical determinations can be performed simultaneously in one sample. Moreover, because the elemental tag can be made containing many atoms, the measured signal can be greatly amplified.

The use of multiple copies of the element or isotope per tag can improve the sensitivity linearly, particularly, for example in the employment of ICP-MS embodiments of the invention. For multiplex assay of up to 23 simultaneous analytes, the tags can be conveniently constructed using the natural isotopic distributions of, for example, Ru, Rh, Pd, Ag, In, La, Ce, Pr, Nd, Sm, Eu, Th, Dy, Ho, Er, Tm, Yb, Lu, Hf, Re, Ir, Pt and Au. These elements, which are expected in most instances to be uncommon in biological samples, each have at least one isotope with natural abundance greater than 10% that is not significantly interfered by the others or by the oxide or hydroxide ions of the others. For those isotopes of lower natural abundance (e.g., ^{143}Nd, 12.2%), tagging with the isotopically enriched isotope provides an obvious sensitivity advantage. Where a higher order of multiplexing is desired, the use of commercially-available enriched isotopes (of which there may be as many as 167 of 55 elements that are not expected to be common in biological systems) offers a possibility (depending, of course, on availability, cost and isotopic purity). For example, there are as mentioned at least 35 isotopes of the lanthanides and noble metals alone that may be obtained in enriched form, are note expected to be common in biological systems and are largely independent with respect to mutual interference (though some care in the selection of the tagging protocol is to be taken where large differences in copy counts of the analytes occur; for example, if the copy count of an analyte tagged with ^{169}Tm is 1000 times greater than for an analyte tagged with ^{185}Er, $^{169}TmO^+$ will interfere significantly with the determination of $^{185}Er^+$ since TmO^+ is typically about 0.07% of the Tm^+ signal (though, as for FACS, some of this interference can be corrected mathematically since the fractional formation of oxide ions is stable and can be calibrated). In special circumstances, it might be feasible to tag a given biologically active material with more than one element or isotope (for example, there are in theory 20 distinguishable 3-isotope tags that can be constructed from 4 isotopes).

The invention allows the development of a novel powerful technique to measure biological properties of cells by analyzing single cells as they pass through an ICP. When using antibodies as the affinity product (biologically active material) the amount of a tag element detected by the mass spectrometer is proportional to the amount of tagged affinity product bound to the cell. Antibodies conjugated to the elemental tag are routinely used as reagents to measure the antigen both qualitatively and quantitatively, for example acquiring the patient's immunophenotypic profile, which is almost unlimited in the number of markers of interest. Another advantage offered by the invention is a reduced need to enhance the antibody signal by "sandwich" immunostaining" which can result in analytical errors.

Methods according to the invention are distinct from the approach of conventional methods (such as fluorescence, radioimmunoassay, chemiluminescent assay) that are challenged by overlap of detector signals, limited dynamic range, time-sensitive signals, and in some instances sensitivity. Accordingly, the method offers the potential for massively multiplexed assay (limited principally by the independence and cross-reactivity of the affinity chemistry) with essentially no signal overlap. Where the elemental (isotopic) tags are quantitatively associated with specific affinity products, the quantitative characteristic of ICP-MS offers a novel opportunity for absolute determination of multiple antigens simultaneously.

The method and apparatus can, for example, detect as few as 100 copies of each tag per cell. It is estimated that for the detection of as few as 100 copies of each tag per cell, at least 70 atoms per tag will be required.

The invention provides the feasibility to perform massively multiplexed bead assays. Current fluorescence-based flow cytometers are frequently used for bead assays. In this application, beads are typically labeled with 2 fluorochromes in varying ratios, typically providing up to about 100 distinguishable beads as determined by the fluorophore emission ratio (see, for example, the incorporated reference, U.S. Pat. No. 6,524,793 and references therein). Each bead also has attached affinity products (e.g., antibodies) that recognize an analyte in a solution in which the bead is placed, each bead of different "colour" having an affinity product for a different analyte. Once exposed to the sample solution, the captured analyte is then sandwiched with another antibody having a third fluorophore reporter. Thus,

in the flow cytometric analysis, the beads can be mixed, the copy-count of the analyte captured determined by the emission of the third fluorophore and the identity of the analyte determined by the ratio of the emissions of the bead-labeling fluorophores. Accordingly, the conventional fluorescence detector flow cytometer can perform multiplexed bead assays to as high 100 order (the number of distinguishable "colours", though in practice much fewer are used (presumably because of signal overlap, which limits the measurement accuracy (and thus confidence in the identification of the bead) when the ratio of the fluorescence emission intensity is large (e.g., one or two orders of magnitude, depending on the emission wavelength distributions).

A similar method can be implemented using mass spectrometer-based flow cytometers according to the invention, with the advantage that the degree of multiplexing can be vastly increased and the overlap of signals can be virtually eliminated from concern. For example, the bead can incorporate (either on its surface or, probably more conveniently, within its body, mixtures of elements or isotopes that can be used to report the identity of the bead. Assuming that the detector has a dynamic range of 3 orders of magnitude and that factors of 3 in relative signal can be reliably determined, 2 elements incorporated into the bead allows 63 distinguishable beads. Under the same assumptions but using 5 element labels provides 32,767 distinguishable beads, and if the dynamic range is 5 orders of magnitude, the same 5-element labels provide for 248,831 distinguishable beads. Furthermore, these few labeling elements can be selected so that signal overlap is nonexistent (e.g., by choosing them such that they appear at mass differences greater than a few atomic mass units), which enables the large dynamic range of detection. The sandwich assay for the analyte captured by the bead employs a yet different element tag, which also is readily distinguished from the bead-labeling elements. Further, in this configuration each bead can contain several affinity products to attach several different analytes per bead, each recognized by a sandwich assay using a yet different element, providing for multiplex assay both between beads and on a single bead. One anticipated application is for a 96-well plate (or 384-well, or 1536-well) for which a differently-labeled bead is provided to each well, and multiplexed element-tagged immunoassay on the bead surface in each well is conducted. The entire contents of the plate (96, or 384 or 1536 wells) can then be pooled and the result analyzed by flow cytometry, thus providing a type of mass spectrometer "plate reader" (where the bead identity, as determined by its elemental composition, identifies the well in which the assay took place).

Means for Introducing Particles Sequentially

The sample introduction system **102** can comprise several devices that are currently in use with other flow cytometry sample introduction systems. For example, there currently exist several cell or particle injector **171** systems in use for flow cytometry, including various formats of sheath flow injection. Because of considerations for solvent loading of the ICP (typically optimum for 25 to 80 μL/min), the "flow in air" (or in the instance of the ICP, "flow in argon") injector **171** may in some circumstances be considered most appropriate (though some improvement over current designs may be preferred, in order to minimize cell agglomeration). All sample introduction devices suitable for the purposes disclosed herein; including ICP devices, will serve, regardless of whether they now exist or are hereafter developed or improved.

For the feasibility experiments that we report below, a small volume spray chamber (similar in concept to a design reported by J. L Todoli and J. M Mermet, J. Anal. At. Spectrometry 2002, V17, 345-351) was employed, having a drain to remove condensed liquid (of which there was essentially none at the suspension flow rates used) and having no gas outlet except into the ICP.

It is noted that, compared to the FACS method for which careful alignment of the particles with the excitation laser is important, the present method allows relaxation of the alignment of the particles with the vaporizer, atomizer and ionizer (unless light scattering is used as the particle detection trigger; see later). This is because, especially for the ICP instance, the precise position of the particle within the injector tube feeding the ICP is of little importance to the detected signal (in part because the central channel flow containing the particle expands dramatically upon heating and in part because virtually all of the central channel flow is inhaled into the sampler of the ICP-MS vacuum interface, though only the predominantly central portion is subsequently transmitted through the skimmer; in any event, there appears to be substantial mixing of the central channel flow prior to sampling into the vacuum interface).

It is desirable that the entire particle introduced to the ICP be vaporized, and at least partially atomized and ionized, so as to enable determination of the element tags contained within the particle (intracellular tags, or bead labels). Current wisdom holds that solid particles (e.g., of glasses or geological materials) smaller than about 1 μm diameter, and liquid aerosols smaller than about 10 μm diameter, are efficiently vaporized, atomized and ionized in the ICP, while larger particles may be only partially volatilized. This is presumably due to the short transit time of the particle through the ICP, for which the heat transfer to a large particle is insufficient to allow complete vaporization, atomization and ionization. Thus, it is propitious to use beads having a diameter smaller than about 1 μm diameter (for example, we used stober silica particles of about 150 nm diameter in our feasibility studies described below). However, cells are frequently larger than 10 μm diameter. Nonetheless, our feasibility experiments, described below, suggest that cells larger than this perceived minimum are, in fact, efficiently vaporized, atomized and ionized, from which we infer that, upon the rapid heating during transit through the ICP, the cell explodes into fragments that are small enough to be vaporized, atomized and ionized. It remains possible that in certain instances the particles may be too large to allow efficient vaporization, atomization and ionization, which could be indicated by the failure to observe an intracellular tag or the element labels of a bead. In this instance, several ion source parameters (gas flow, power, sampling depth) can be adjusted to alleviate this deficiency. Alternatively, an in-line lysis component can be employed.

In-line Lysis

In-line lysis system **110** may be advantageously employed in some circumstances. For example, in the event that whole cell introduction is not viable, use of an in-line lysis system can be advantageous. This may be done by any method suitable for the purposes disclosed herein, including a number of methods now known to persons skilled in the art, including acidification of the sheath flow fluid to cause cell collapse or high purity (low conductivity) water sheath flow to induce rupture of the cell by osmotic pressure. In this instance, the elemental tags will be retained and transmitted to the device to vaporize, atomize and ionize the sample, though the transient pulse may be broadened slightly by diffusion in the flow stream.

Means for Vaporizing/Atomizing/Ionizing

Any means **104** suitable for the purposes disclosed herein can be employed to vaporize, atomize and excite or ionize the particle or the elemental tag associated with the particle; for example, graphite furnace, glow discharge and capacitively coupled plasma. Preferably, the vaporizer/atomizer/ionizer is an inductively coupled plasma. In some instances, vaporization, atomization and ionization and/or excitation can occur in different devices and at different times (e.g., within a graphite furnace for vaporization in combination with ICP for atomization and ionization and/or excitation.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) is a preferred means of determining the elemental composition, especially ultra-trace components, of materials. It has found acceptance in various applications including environmental (e.g., drinking, river, sea and waste water analyses), geological (e.g., trace element patterning), clinical (e.g., determination of trace metals in blood, serum and urine) and high purity materials (e.g., semiconductor reagents and components) analysis.

ICP-MS couples an inductively coupled plasma ionization source to a mass spectrometer. Briefly, a sample, most commonly an aerosol produced by nebulization, is injected into a high temperature atmospheric pressure plasma obtained by the coupling of radio frequency (rf) energy into the flowing argon gas stream. The resultant plasma is characterized by a high temperature (ca. 5000K) and relatively high concentration (ca. 1015 $cm.^{-3}$) of equal numbers of electrons and positive ions. Provided that the particles of the nebulized sample are small enough, as described above, the sample is promptly vaporized, atomized and ionized as it flows through the plasma. The efficiency of ionization is inversely and exponentially dependent on the ionization potential of the elements, with the majority of the periodic table being nearly 100% ionized. The resultant plasma containing the ionized sample components is extracted into vacuum where the ions are separated from neutral species and subjected to mass analysis. The "mass fingerprint" identifies the elements contained in the sample. The detected signal is directly and quantitatively proportional to the concentration of the elemental composition of the sample. The particular attributes of the method of note include: wide linear dynamic range (9 orders of magnitude), exceptional sensitivity (sub-part per trillion, or attomole/microliter, detection), high abundance sensitivity ($<10^{-6}$ overlap between adjacent isotopes for quadrupole analyzers), counting-statistics-limited precision, absolute quantification, and tolerance of concomitant matrix.

ICP-OES is another preferred method of performing the analyses described above; it is of particular merit when the solids content of the sample is greater than about 1% (for homogeneous liquid introduction rate of the order of 1 mL/minute). The conditions employed in the ICP are comparable to those described for the ICP-MS method. Detection of the emission from excited neutral atoms and ions in the ICP provides for the quantitative determination of the elemental composition of the sample. Most current ICP-OES instruments provide array detection for true simultaneous determination across most of the periodic table. In many favorable instances, ICP-OES retains some of the desirable characteristics of ICP-MS, including wide dynamic range and well-resolved detection channels. In other instances, there is potential for inter-element or molecular emission interference, though in such instances alternate emission wavelengths are frequently available. The principal deficiencies for the application considered here are its generally lower sensitivity (in some instances limited by background emission signals) and its inability to distinguish isotopes of a given element. Nonetheless, ICP-OES is perceived to be more simple to use, more robust, and less expensive than ICP-MS, and hence may have application for the present method.

Ion Pretreatment Device

In some circumstances, as for example in MS FC, an ion-pretreatment device **112** may be used to condition the ions for the mass analyzer. Because the mass spectrometer operates at reduced pressure (typically less than 10^{-4} torr) and the ion sources noted above typically operate at higher pressure (e.g., atmospheric pressure for the conventional ICP), one function of the ion-pretreatment device is to efficiently transport the ions derived from the sample through a pressure reduction step (the vacuum interface). It is desirable in this step, and subsequently, to increase the ratio of ions to neutrals that are subsequently transmitted to the mass analyzer. Ion optical components (ion lenses) typically serve this function, by localizing the ions and allowing the neutrals to be removed through vacuum pumps. An additional function of the ion optics is to condition the ion beam (in space and energy) to match the acceptance of the mass analyzer.

High-pass filter **116** and 'cooler' cells **118** are only two of the many suitable forms of pretreatment that now exist; doubtless other forms will hereafter be developed. Any devices or methods suitable for the purposes herein will serve.

Due to the short residence time of a single particle passing through the plasma, two separate ion handling (pretreatment) and mass separating techniques may be used.

A gain of two orders of magnitude relative to current ICP-TOF-MS instruments, which means about one order of magnitude greater than current quadrupole systems is also desired. The mass spectrometer-based flow cytometer is ideal for the detection of heavy atom tags. It is sufficient to determine only the mass range above ca. 100 amu. One of the most significant impediments to improved sensitivity is space charge repulsion of the dominant Ar^+ ions (m/z=40). Since the method is not limited by the conventional elemental analysis demands (the mass range of the typical elemental analyzer is from m/z=4 to m/z=250), it is possible to optimize the ion optics for the transmission of high mass ions.

While a conventional ICP-MS having simultaneous detection capability (for example, an ICP-TOF-MS **126** or ICP-ion trap-MS) is as a detector of the MS FC **101**, it should be realized that the requirements of the MS FC **101** are quite distinct from those of the conventional elemental analysis application. In particular, in the MS FC application the elements to be determined (as tags or labels) can be selected with advantage to be those above, say, 90 atomic mass units (amu, dalton, Thomson). In such instance, there is no need to provide simultaneously optimum sensitivity for low mass (e.g., Li, B, Na, Mg, Co, etc.) and high mass (e.g., the lanthanides and noble metals).

One approach employing a TOF analyzer **126** is to accelerate the ion beam relatively early in the plasma expansion because an accelerated beam has a higher space-charge-limited ion current and to high-pass filter the beam. This can be through the use of a quadrupole-type device, which is not pressurized. The depleted ion beam can then be decelerated (even collisionally cooled in a pressurized multipole, which could potentially also provide ion-bunching) prior to injection into the TOF. It is anticipated that the space-charge limit of such a continuous extraction beam is

sufficiently high to allow a ten-fold improvement in sensitivity for the higher mass ions.

An alternate, or concomitant approach, is to pulse-extract the ion beam. Since lower mass ions are accelerated to high velocity in a given extraction field, the Ar^+ ions (and lower mass ions, which can be discarded) run ahead of the higher mass ions of interest. Preferably, the front-running ions can be discarded using an orthogonal pulse, similar to "SmartGate" of the GBC TOF, (see, e.g., WO 98/33203) but in the ion optics region. The transmission window does not need to be precisely defined in this instance, as it is sufficient to intercept ions <100 amu. A downstream cooling cell could still be used to bunch the ions and normalize their energies. If the orthogonal pulser is problematic, the entire pulse-extracted ion beam can be run into the TOF extraction region, with the deficiency that more-narrow mass windows will be simultaneously injected into the TOF. Simple calculations (which overestimate the potential by at least some margin) indicate that a 15% duty cycle pulse-extractor could yield up to 28-fold (m/z=100) and 12-fold (m/z=238) sensitivity improvement over current (80 Mcps/ppm) quad systems. This assumes 100% transmission efficiency through the ion optics and 100% duty cycle of the TOF (requiring bunching).

The ion pretreatment device may also include a particle event trigger, which triggers instrument mass selection and detection systems to acquire data from discrete particles, and keeps the instrument idle between events. As is known to those skilled in the art, this can be done in many different ways.

Therefore, the ion pretreatment device may comprise:

a vacuum interface;

a high-pass mass filter downstream of the vacuum interface; and

a gas filled ion cooler cell downstream of the vacuum interface.

Among the distinctions that simplify the design of the MS FC **101** according to the invention relative to a conventional elemental analyzer are the relative invariance of the sample (cells or beads in a known buffer) that simplify the need for an ionizer design (e.g., ICP) that is tolerant of various sample types and matrices, the relative (with respect to the total ion current of the ICP) invariance of the total elemental composition of the sample that relieves the need to provide compensation for inter-element matrix suppression effects (recognizing that, for example, Na and Ca will be significant components of cells), and to a large extent (depending on the selection of tag elements) the need to compensate for the presence of spectral interferences due to argides, oxides and doubly charged ions. Thus, MS FCs according to the invention can be advantageously adapted to suit the cytometric application but not for the general elemental analytical application because of the selectability of the elements to be determined. For example, conventional elemental analysis by ICP-MS is compromised by the mutual repulsion of ions following extraction into the vacuum system; this space charge effect, well known to those skilled in the art, derives principally from the overwhelmingly large flux of lower mass ions that derive from the plasma support gas or the sample solvent such as O^+, Ar^+, ArO^+, $Ar2^+$, and in some instances lower mass ions that derive from other sample matrix components such as Na^+, Ca^+, Cl^+. It will be recognized that the most significant of these ions that form the bulk of the space charge effect are low mass ions, being below about 80 amu. Thus, advantage is to be had by eliminating such low mass ions as early as possible following extraction into the vacuum system because doing so will alleviate the space charge and its associated effective potential field barrier that suppresses transmission of other ions. Several schemes for achieving this relief can be conceived, including the use of a high-pass mass filter such as a quadrupole device that is operated to transmit ions above, say 80 amu. Notably, a quadrupole can be operated at the pressures extant in the ion optics region (typically about 10^{-3} torr). An additional advantage of such an ion pretreatment device for the present application is that it can also be operated to simultaneously provide a low pass mass filter function (that is, a bandpass between a selected low mass and a selected high mass). In the instance that a time-of-flight mass analyzer is used, this bandpass can provide an improvement in duty cycle (resulting in improved sensitivity) because it minimizes the incursion of the arrival of high mass ions from a previous pulse into the arrival time distribution of the current pulse and also the incursion of low mass ions from the previous pulse into the arrival time distribution of the current pulse (where "pulse" means the packet of ions that are injected into the flight tube of the time of flight mass analyzer). Further, acceleration of the ions as soon as possible upon their entrance to the vacuum system (or near the point where the debye length of the plasma is comparable to the dimensions of the apparatus or lenses) can further mitigate the space charge effects. However, in the instance that the ions are subsequently decelerated (for example, in the acceleration region for the TOF), the space charge effects can return and reassert themselves resulting in reduction of sensitivity and, in the instance of the TOF, reduced mass resolution due to energy broadening in the direction of the flight tube. Hence, the high pass mass filter, which can be functional at relatively high ion kinetic energy if appropriately designed, can be operated in concert with acceleration optics to mitigate space charge effects both immediately downstream of the vacuum interface and further downstream, for example in the acceleration region of a TOF mass analyzer **126**.

It is further advantageous, as is well known to those skilled in the art, that reduction of the axial ion energy by collisions with a non-reactive buffer gas in a pressurized multipole cell (a "cooler" cell) **118** provides improved resolution and sensitivity for TOF mass analysis (also expected to be true for an array-detector magnetic sector mass analyzer). Here again, the high pass mass filter **116**, which should precede the "cooler" cell **118**, can be operated in concert with the "cooler" cell **118** with advantage, since bandpassing the ions prior to the "cooler" cell **118** will mitigate to large extent space charge effects that otherwise would be detrimental (i.e., cause loss of sensitivity) in the "cooler" cell (which would happen because the ions are slowed by collisions in the "cooler" cell, and slowing them without first removing the bulk of the low mass "space charge inducing" ions causes an abrupt appearance of a significant defocusing space charge field near the entrance of the "cooler" cell).

As is known to those skilled in the art, in certain instances advantage is also to be had in including reactive gases in the "cooler" cell **118** in order to transform ions that are isobaric and thus are interfering or are interfered (reference U.S. Pat. No. 6,140,638). Further, the "cooler" cell can also be operated in a trap-and-pulse mode that could be optimized for synchronous operation with a TOF acceleration pulse to provide improved duty cycle (and hence sensitivity) for that mass analyzer. Thus, the MS FC **106** can incorporate with advantage ion acceleration optics and a high pass mass filter.

For several mass analyzer embodiments, including in particular the TOF and array-detector magnetic sector con-

figuration, the use of a gas-filled "cooler" cell is also advantageous. For the TOF configuration in particular, the high pass mass filter could with advantage be operated as a bandpass mass filter with both a low and a high mass transmission limit. As is known to those skilled in the art, the high pass mass filter and "cooler" cell can be combined as a single unit (cf., U.S. Pat. No. 6,140,638).

Advantage is also to be had, to minimize the volume of data collected to include only the most significant data or, in the instance of a mass analyzer (such as TOF) which is constrained by a duty cycle, to coordinate the measurement of data with the passage of a particle of interest through the detector system. In the conventional FACS method, this coordination is accomplished most often by the measurement of light scattering as the particle passes through the excitation region; the nature of this light scattering (forward and side light scatter) can provide information on the size and granularity of the particle which also has diagnostic value. In the MS FC or OES FC method, light scattering can be similarly used.

Where the source of excitation is an ICP, the scattering event can be detected prior to vaporization of the particle; hence a delay corresponding to the time or spatial delay required for signal generation. For OES FC this is the time or distance required for vaporization, atomization, ionization and emission; for MS FC an additional delay corresponding to the transit time of the extracted ions from the region of ionization to the mass analyzer is required. Those skilled in the art will realize that for continuous monitoring mass spectrometers, for example an array detector magnetic sector mass analyzer, this delay should be applied to the arrival of the ions at the array detector. For other mass analyzers, for example TOF and ion traps, the delay is applied to the device that introduces the ions into the mass analyzer, for example the acceleration region preceding the flight tube of the TOF or a pulsing lens that introduces ions to an ion trap, to which the subsequent mass analysis and detection is synchronized.

Other methods of providing a trigger for data collection are contemplated for the MS FC **106**. For example, it is expected that the passage of a particle through the ionizer (for example, the ICP) will cause an abrupt and consequent change in the mass distribution of the major ions that are extracted (for example, the dominant Ar^+ signal in ICP-MS could be suppressed with concomitant formation of C^+, $^+$, Na^+, Ca^+, etc.). It is thus expected that the ion current ejected or the spatial position of this ion current ejection (due to differences in the stability characteristics of ions of different masses) from, for example, the high pass mass filter, will change significantly and can be detected with one or more electrodes within or external to, for example, the high pass mass filter. Further, the magnitude or duration of the current change detected may be correlated with the size or content of the particle and could provide further diagnostic information.

Other trigger devices are contemplated, including, for example, a detector that measures changes in the ion current or impedance or magnetic field associated with the ion beam extracted into the vacuum system.

Optionally, various components, including for example a high mass filter and a gas-filled ion cooler, may be provided in a single housing. This can provide, for example, improved durability, as well as improved operating, handling and installing qualities.

Mass Spectrometer

The pretreated ion cloud may be analyzed with a simultaneous mass analyzer. Sequential mass analysis (e.g., through the use of quadrupole devices) is also possible. Examples of simultaneous mass analyzers include TOF, 3D trap and Linear Trap.

In some instances where the MS FC **101** method is to be used to best advantage (e.g., multiplex assay of individual particles), a simultaneous mass analyzer is preferred. For example, in the instance of the use of an ICP as the vaporizer, atomizer and ionizer, the transient signals from a single particle may last for a period in the range 20 to 200 microseconds, which can be insufficient to allow quantitative multiplex assay using a sequential mass analyzer, for example a quadrupole mass analyzer. In such instances, examples of preferred mass analyzers include TOF, array-detector magnetic sector, 3D ion trap and linear ion trap. In other instances where the period of the transient signal is significantly longer, either by the nature of the device to vaporize, atomize and ionize or by broadening of the transient signal, for example through transport of the vaporized particles, atoms or ions through a length of tubing or through collisional processes (such as those reported by D. R. Bandura, V. I. Baranov and S. D. Tanner in J. Anal. At. Spect. 2000, V15, 021-928), a sequential mass analyzer may find utility.

At the current state of development of mass analyzers, the TOF appears to be best-suited for the MS FC application. Ion traps (3d and linear) might be suitable provided that they are preceded by a selection device, for example a high pass mass filter, that reduces the space charge in the trap. The array-detector magnetic sector analyzer, which offers high duty cycle and should provide high sensitivity, could be suitable provided that an efficient array detector is developed, though at the present state of development the abundance sensitivity (overlap of signals onto neighbouring mass channels) is limiting.

The most commonly-used mass analyzer **106** coupled to the ICP is at present the quadrupole, principally because of its robustness, ease of use, and moderate cost. However, the quadrupole is a sequential scanning analyzer having a cycle time for multiplex analysis that is long relative to the duration of a transient signal from a single particle in the plasma source. Therefore, the quadrupole cannot deliver correlated multi-analyte signals for such a short transient. A quadrupole ICP-MS analyzer is often used for the analysis of samples presented in quasi-continuous flow, for example for nebulization and laser ablation. It is appropriate for the analysis of homogeneous samples, such as for many conventional immunoassays where total element signaling is of interest.

In contrast, the time-of-flight (TOF) analyzer **126** shown in FIG. **2**, which samples a packet of ions in a given time period and spreads them in time according to their velocities in a potential field which are a function of the mass-to-charge ratios of the ions, is a "simultaneous" analyzer that is suited to the analysis of short transients such as those produced by single particles. Although TOF analyzers are known, the inventors are unaware of any TOF or other mass spectrometer analyzer currently being used for flow cytometry. Commercial ICP-TOF-MS instruments are some 10-100 times less sensitive than quadrupoles, at least in part due to more significant space charge effects in the ion optics and TOF acceleration region and to inefficiencies in duty cycle. With the employment of appropriate ion optics and other concepts noted herein, these deficiencies should be alleviated.

Another useful cytometer configuration is the OES FC **151** shown in FIG. **3**. A distinction between the OES FC **151** and the MS FC **101** is that in the former, light emitted by

both atoms and ions derived from the vaporized particle are collected and transmitted to an optical spectrometer having an array detector. In the ICP embodiment of OES FC **151**, the emission may be collected either radially through the ICP at a specified "height" above the rf load coil (the preferred observation height is a function of the plasma conditions, but is stable for stable ICP conditions) or axially by looking "down" through the plasma towards the injector (which requires a cooled viewing interface usually with a curtain flow of gas), as shown in FIG. **3**. The configuration and use of radial- and axial-viewed ICP-OES instruments is well known by those skilled in the art.

Among the distinctions of cytometers according to the invention from conventional fluorescence-based flow cytometry are that: (1) the cells or beads or analytes are tagged with elements rather than fluorophores; (2) the cells or beads are vaporized, atomized and (optionally, but usually naturally under optimum conditions) ionized and it is the elemental components of the cells and beads that are detected; (3) excitation to induce emission is gained from the ICP (convective and/or electron impact heating) rather than laser excitation at an absorption band of the fluorophore; (4) almost all elements of the periodic table are excited to emission (either atomic or ionic) under the operating conditions of the ICP, whereas multiple fluorophore excitation in conventional flow cytometry generally requires two or more excitation lasers, each of which may excite one or more fluorophores with absorption bands that are coincident with the wavelength of the excitation laser; (5) the emitted light is dispersed by, for example, eschelle gratings or prisms in one or preferably two dimensions and collected on an array of detectors, for example a CCD "camera", whereas the conventional flow cytometer uses bandpass optics to select a "least interfered" wavelength for each fluorophore; and (6) the emission wavelengths are more narrow in ICP-OES than in fluorescence-based flow cytometry, and there is usually more than one usable and detectable wavelength so that inter-element interferences are both less common (better resolved emission spectra) and more easily circumvented (by choosing an alternate emission wavelength).

EXAMPLES

Example 1—Development of Aptamers for Specific Labeling Leukemic Stem Cells

Leukemic stem cells and their progenitor cells can be purified [10]. They can be used as targets for selection of aptamers by selecting for the stem cell and against the progenitor cells using a novel method of combinatorial screening. The selected aptamers can be tested for, and selected against cross-reactivity with other aptamers directed for the multiplex assay of the challenge. The aptamers can be labeled with distinguishable stable isotopic elemental tags as is known to those skilled in the art.

Example 2—Preparation of Labeled MO7E Cell Line

A homogeneous MO7E cell-line which has been transduced with the p210 bcr/abl tyrosine kinase fusion protein from chronic myeloid leukemia can be used. This cell expresses the CD33 surface marker as well contains large amounts of p210 internally. The markers can be tagged with antibodies or aptamers suitably tagged with commercially-available tagging kits (NanoGold™, DELFIA™). The tagged affinity products can be incubated with fixed, permeabilized cells.

Example 3—Preparation of Quadrupole ICP-MS-based Flow Cytometer

Demonstration of concept can be achieved using a quadrupole-based ICP-MS and the tagged cells of Example 2.

A flow cell can be constructed based on a direct injection nebulizer or a sheath-flow non-ionizing nanosprayer. A commercial flow cytometer can be used, but with modifications, and excluding parts related to fluorescence.

Single ion monitoring at the mass/charge of one of the tag elements has improved duty cycle relative to scanning mode so that many of the cell events are, observed. Subsequent measurement, in the same sample but at a later time, at the second surface tag element mass/charge will confirm independence of the affinity chemistry and detection, with the implication that simultaneous determination with an appropriate (TOF) detector is possible. Observation of the internal protein marker will provide important evidence that cell volatilization is achievable. If the internal marker is not detectable, in-line lysis can be used.

Example 4—Development of a Prototype Single Particle Injector

Referring to the injector **171** shown in FIG. **4**, the injector is used to inject cells (or beads or other particles) **400** together with the buffer solution into the desolvation chamber **403** surrounded by a heater **405**. The buffer solution flow is nebulized by high-pressure gas. The volatile component of the buffer and cells (mostly water) is transferred from aerosol to gas phase during the desolvation process and is expelled out of the desolvation chamber together with most of the nebulizer gas through exhaust vents **407**. In most cases, the nebulizer gas flow is limited by size and design of the injector (nebulizer). Therefore, some makeup gas can be introduced to allow complete desolvation. Desolvated heavy cells (or beads) escape directly into the straight cylindrical channel **409** with the rest of the gas and are introduced into vaporizer/atomizer/ionizer **104** of the EFC. In an embodiment the vaporizer/atomizer/ionizer is the ICP plasma, which allows .˜1 l/min of gas to be introduced. Therefore, by adjusting the gap **411** between the desolvation chamber and the cylindrical channel housing, one can control desolvation as well as flow into the ICP plasma.

Example 5—Preparation of ICP-TOF-MS-based Flow Cytometer Research Prototype Instrument

ICP-TOF-MS instruments are commercially available. The TOF mass spectrometer provides a simultaneous analyzer which is beneficial for mulitvariant analysis, of for example, rare leukemic stem cells.

An ICP-TOF-MS can be outfitted with a flow cell. The components of the instrument as shown in FIGS. **1**, **2**, and **3** and described in the preferred embodiment can be assembled. Relevant components of commercial products (ELAN® ICP-MS and prOTO® orthogonal MALDI-TOF) can be procured as the basis of a working system. Some modification of the operating system will be required to address the specific data collection issues of the cytometer prototype; suitable modifications are well understood by those of ordinary skill in the art. It can be sufficient to operate independent computer control systems for the ICP

source and the TOF analyzer, as this would allow rapid and efficient research investigation.

An instrument can be evaluated with respect to its analytical performance for homogeneous aqueous sample introduction as well as homogeneous cell digests. The ICP-TOF MS-based flow cytometer can be tested, for example, using human established leukemia cell lines (MO7e, K562, HL-60) to investigate the capabilities with respect to the needs for the cytometric application. Specifications for dynamic range, abundance sensitivity, transient signal pulse width and detection mode (analog/digital) for the research prototype instrument can be established.

The following examples have been demonstrated using a conventional quadrupole ICP-MS instrument (sequential scanning) using conventional nebulization of solutions obtained by acidification with HCl of the sample following immunoprecipitation and washing, which digested the sample yielding a relatively homogeneous solution. Thus, "simultaneous" determination refers in this instance to simultaneous immunoprecipitation followed by sequential measurement of the concomitant tags by ICP-MS.

Example 7—Dynamic Range of Anti-Flag M2 Agarose Bead Element-Tagged Immunoassay

FIG. **5** is a calibration curve of the ICP-MS linked immunoprecipitation assay of 3.times.FLAG-BAP. M2 agarose beads were used to capture samples of serially diluted 3.times.FLAG-BAP over a concentration range of 0.05 ng to 1500 ng per 100 μl 3×FLAG-BAP was detected using an anti-BAP primary antibody and an anti-mouse-nanoAu secondary antibody. Diluted HCl was used to dissolve the nanogold tag for ICP-MS sampling. The results indicate that the detected signal (for gold) is linearly proportional to the antigen (FLAG-BAP) concentration, and that at least 4.5 orders of magnitude of linear dynamic range are achievable. Large dynamic range is important in the cytometric application to permit simultaneous determination of biomarkers that appear in largely different copy-counts per cell or bead.

Example 8—Simultaneous Assay of Two Cytokines Using Beads

Fluorokine™ beads coupled with cytokine capture antibodies against either TNF-α. or IL-6 were mixed and exposed to a mixture of cytokines, including TNF-α. and IL-6, incubated and then probed with cytokine-specific antibodies tagged with Eu (for anti-TNF-α.) and Tb (for anti-IL-6). After washing and digestion with HCl, the solution was analyzed for Eu and Tb. FIG. **6** provides calibration curves derived from this simultaneous immunoassay experiment. Linearity of signal with antigen concentration over at least 3 orders of magnitude is observed.

Example 9—Simultaneous Assay of Two Proteins Using ICP-MS-Linked Maleylation Immunoassay

FIG. **7** shows the simultaneous quantitation of two proteins using a direct immunoassay conducted in a Reacti-bind Maleic Anhydride 96 well plate, coupled to ICP-MS detection. In this experiment, two proteins (Human IgG and 3×FLAG-BAP) in 1×PBS were incubated in triplicate for one hour at room temperature to allow binding to the surfaces of the well of the Maleic Anhydride plate. Negative controls consisted of 100 μl PBS without protein. The plate was probed with primary antibodies anti-Human Fab'-nanoAu and anti-FLAG-Eu, washed and acidified with 10% HCl with 1 ppb Ir and 1 ppb Ho as internal standards [11]. Homogeneous samples were used wherein the elemental tag(s) are released to acidic solution for conventional nebulizer introduction to the ICP-MS. Note that the sensitivity to IgG using the nanogold tag is approximately 10 times greater than that for FLAG-BAP using the Eu tag; this is because each nanogold tag contains approximately 70 gold atoms (Au is monoisotopic) while each Eu tag contained only between 6 and 10 Eu atoms, approximately equally distributed between the two natural isotopes of Eu (^{151}Eu and ^{153}Eu, the sum of which were measured). The example demonstrates that at least two proteins can be immunoreacted simultaneously and detected without mutual interference, and that the sensitivity scales with the concentration of the antigen and with the number of atoms of the measured isotope per tag.

Example 10—Preparation of a Kit for the Analysis of an Analyte Bound to a Single Cell by Mass Spectrometry

A kit is assembled comprising (1) a tagged biologically active material which binds to an analyte of interest bound to a single cell and (2) instructions for single cell analysis by mass spectrometry.

Example 11—Forensic Applications

The methods and apparatus of the present invention can be used for forensic applications. For example, the methods and apparatus can be used to:

determine antigenic blood types (ABO and Lewis types);
identify body fluid (blood, semen, saliva) and other biosamples (whole blood, plasma, serum, urine, cerebrospinal fluid, vitreous humor, liver or hair);
determine tissue origin (species, personal identity, etc.);
determine paternity.

Example 12—Transfusion Medicine

The methods and apparatus of the present invention can be used in transfusion medicine to:

resolve blood group A, B and D typing discrepancies;
determine the origin of the engrafted leukocytes in a stem cell recipient; and
determine the origin of lymphocytes in a patient with graft-versus-disease.

Example 13—Flow Cytometer with ICP-MS Detector Feasibility Test

We have performed feasibility studies to validate the concept of the present invention. A quadrupole (sequential mass scanning) ICP-MS instrument designed for conventional elemental analysis (and thus not optimized for the flow cytometric application) was used. The instrument was modified in only two ways: a modified sample introduction system was installed, and an oscilloscope was attached in parallel with signal handling hardware and software of the original detector system.

The sample introduction system **102**, **800** is shown schematically in FIG. **8**. Sample **400** consisting of cells or other particles was aspirated using a syringe pump connected with capillary tubing **801** to a small volume spray chamber **803** having a drain **805** to remove condensed liquid and having no gas outlet except into the ICP through the 2 mm diameter injector tube **807**. Sample was pumped at 50 μL/min, about

half of which was drained from the spray chamber **803** and half delivered to the ICP. The inventors recognize that this sample introduction device **800** may not necessarily optimum for presentation of single particles to the ICP with high efficiency in all cases, depending upon the circumstances of the analysis, but it was sufficient in this case to introduce at least a fraction of the particles into the ICP and thus to show feasibility.

The discrete dynode detector of the ICP-MS instrument provides signals that are either analog or digital (pulse). The analog signal, taken part way along the dynode chain, is converted to digital output in the hardware and software of the detector system. The digital (pulse) signal is taken from the final dynode of the chain, is amplified and transient signals corresponding to single pulse events whose amplitudes exceed a given threshold are counted in the detector system hardware and software. The detector system hardware and software can be configured to provide output not of each pulse, but the integral of these over a specified measurement period (minimum about 100 microseconds). In normal operation, if the signal detected at the analog dynode exceeds a specified threshold, the dynode chain downstream is disabled (disabling digital signal detection). If the analog signal is higher than a second threshold, the detector firmware adjusts the voltage of the ion optics of the instrument to defocus the ions from the detector in order to protect the detector. An oscilloscope was tapped into the analog output and operated in parallel with the detector hardware to enable the measurement of the transient events over the period of a single particle event in the ICP (e.g., up to several milliseconds with as low as a few nanoseconds resolution).

Because the instrument used for these experiments is not capable of measurement of more than one mass/charge channel during a short transient period, multiplex analysis of a single particle event in the ICP was not be demonstrated. However, measurement of single mass/charge detection channel events has allowed demonstration and evaluation of certain important characteristics of the ICP-MS detector system for the cytometric application. The inventors believe that these characteristics can be replicated, with some differences depending on the selected embodiment of the instrument configuration, with a simultaneous mass analyzer, with the additional benefit of facilitation of simultaneous measurement of many mass/charge detection channels permitting multiplex assay of single particles.

Feasibility Test 1: Detection of Single Particle Events, and Estimate of Sensitivity of Current Instrument

The MO7e cell line is a human megakaryocytic leukemia-derived cell. MO7e expresses CD33 antigen (67 kDa single chain transmembrane glycoprotein, myeloid cell surface antigen CD33 precursor (gp67)). The cell is thought to express approximately 5000 to 10000 copies of antigen per cell. The cell line was used to demonstrate that individual cells can be observed by methods according to the invention, and to estimate the sensitivity of such method using the current instrumentation. The CD33 surface marker was detected using monoclonal anti-CD 33 (IgG1 mouse) and Nanogold™-tagged anti-mouse secondary antibody (approximately 70 Au-atoms per tag). It is estimated that the efficiency of secondary antibody staining is approximately 10%.

Materials

MO7e cells were cultured for three days in a T75 flask. The cell concentration was determined by hemocytometer and found to be $0.5.\times10^6$ cells/ml.

Monoclonal antibody anti-CD33, unconjugated. IgG1 (mouse) isotype supplied at 2 mg/ml and purified in PBS/BSA with 0.1% sodium azide by Immunotech Inc. Cat#1134.

Secondary anti-mouse IgG conjugated with nanogold from Nanogold Inc. (approximately 70 Au-atoms per tag)

1% formalin prepared from 37% formalin; diluted in PBS.

Wash and antibody dilution buffer PBS/1% BSA.

50 mM ammonium bicarbonate buffer, pH 8.0.

Procedure

Tubes were soaked in PBS/1% BSA for one hour. MO7e cells were pelleted at 1500 rpm (at approximately 200 g) 5 min, resuspended in 5 ml PBS, pelleted and the wash discarded.

Cell pellet was resuspended in 3 ml PBS/1% BSA and distributed into three eppendorf tubes (at approximately 106 cells/tube) marked as primary and secondary antibodies added; only secondary antibody added; or no antibodies added.

Primary antibody was diluted 1:50 in PBS/1% BSA and added to the cell pellet for 30 min on ice.

Cells were washed with PBS/1% BSA once.

Secondary antibody was diluted 1:50 in PBS/1% BSA and added to cell pellet for 30 min on ice.

Cells were washed once with PBS/1% BSA, once with PBS.

Live stained cells were fixed in 1% formalin/PBS for 10 min RT and left in the fixative on ice overnight.

Cells that did not receive antibodies were treated only with PBS/1% BSA concordantly with the stained cells.

Stained formalin fixed cells were pelleted at 1500 rpm (at approximately 200 g) for 5 minutes and resuspended in 1 ml 50 mM ammonium bicarbonate buffer, pH 8.0 per tube next day. This was discarded after centrifugation and fresh bicarbonate (0.5-1 ml) was added to each tube.

Tubes were vortexed gently to break up the pellet, left to sit for 5 minutes for large clumps to settle to the bottom, and the top 25 μL of whole cell suspension were injected into the ICP-MS instrument.

Observations

The integrated (pulse detector) signal for Au for discrete cell introduction gave 300-500 counts per second (cps), secondary antibodies only, less than 100 cps, no antibodies, less than 10 cps and buffer only, less than 3 cps.

FIG. **9** shows the overlaid results of separate direct injections of the 100 ppt Rh (1% HNO3) and cell suspension (separate injection, 50 mM NH4HCO3) samples as described above.

FIGS. **10**A and **10**B show oscilloscope data associated with FIG. **9**. FIG. **10**A (on the left) shows the signal for Ar_2^+ (about 10^7 cps) signal. The upper trace covers a relatively large time window (ca. 100 .μs), from which one could conceivably determine the average ion signal rate. The lower trace shows the pulse for a single ion detection event (over a greatly magnified time scale). FIG. **10**B (on the right) shows Au^+ from cell introduction. The upper trace indicates that multiple ion signal pulses are not observed. The lower trace shows the signal pulse for a single Au ion detection event. Typically, only one ion pulse was observed in a particle event time window, suggesting that we detect on average only about one Au atom per cell.

Efficiency of Detection

We estimate the sample introduction rate at (very) approximately 400 cells/second, derived as follows: approximately 1×10^6 cells per sample, 1 mL/sample at 50 .μL/minute introduction sampling with 25 .μL/min delivered to the ICP.

Accordingly, we infer that approximately one Au atom per cell is observed.

The detection efficiency of the instrument used was estimated from the signal obtained from continuous aspiration of a sample containing 100 parts per trillion (ppt, mass/volume) in 1% nitric acid. The signal obtained was approximately 2000 cps, suggesting an efficiency for detection of Rh of 1×10^{-5}, derived as: 100 ppt (10^{-10} g/mL), atomic weight 103 g/mole, 25 μL/min delivered to plasma, yielding 2×10^{8} atoms Rh/second delivered to plasma for which 2000 cps is observed. The inventors ascribe this efficiency to the following: 100% ionization, 1% transmission through the vacuum interface (100% of central plasma containing ion inhaled through sampler, 1% of sampler flow transmitted through skimmer), 10% transmission through mass analyzing quadrupole, and therefore about 1% transmission through the ion optics. (From these estimates, we infer that improvements in sensitivity for the cytometric application, assuming retention of the vacuum interface configuration, should principally focus on improving the transmission through the mass analyzer (e.g., TOF with high duty cycle) and, more importantly, the ion optics (according to the earlier discussion, principally through accelerating optics and elimination of space-charge-inducing ions)).

Therefore, if one Au atom detection event per cell is obtained, and this is obtained with the same detection efficiency as Rh solution, we estimate that the MO7e cell averages approximately 1400 tagged CD33 markers per cell. With the assumption that the efficiency of the two antibody tagging is about 10%, the estimated number of CD33 per cell is 14000, which is consistent with the 5000-10000 quoted earlier. It is desirable to provide higher sensitivity so that proteins of lower copy-count per cell can be detected. In addition to the ion optical improvements suggested above, direct immuno-tagging (as opposed to the 2 antibody sandwich used here) is expected to be advantageous.

We conclude that the method is able to detect single particle events in the plasma. The experiments described provide guidance for research efforts to improve the sensitivity of the method. A simultaneous mass analyzer is required to facilitate the multiplex advantage that the mass spectrometer detector provides to flow cytometry.

Feasibility Test 2: Estimation of the Transient Period of a Single Particle Event

The MO7e cell sample used in Feasibility Test 1 provides an opportunity for the estimation of the transient period of a single particle event, which is important for the design and optimization of the MS FC. It is estimated that the NaCl content in the cell is 0.9% w/w. For a 16 micrometer cell this converts to 2×10^{11} atoms of Na per cell. The efficiency of the instrument used in these experiments for Na detection is lower than for Rh; about 1×10^{-6}. Thus, for a single cell event, 2×10^{5} ions will reach the detector. This is a sufficiently-large number that the arrival period of Na ions corresponding to a single MO7e cell event can be measured.

If the transient produced by the single cell event is of the order of 100-300 microseconds (as reported by Olesik for monodispersed 3-65 micrometer particles), an equivalent average count rate of $(0.7–2)\times10^{9}$ is achieved (with peak current about twice that).

FIGS. **11**A and **11**B show the Na^+ signal detected at the oscilloscope over the period of several cell introduction events. The data given in FIG. **11**A shows the Na^+ signal when cells are introduced in a 30 mM $CaCl_2$ buffer. The data shown in FIG. **11**B presents the results for buffer only. The variability of the observed signals may reflect the variability in cell size (volume and thus Na content) of the cell population, or might indicate the presence of Na-containing particles other than MO7e cells. The important observation, for the present purposes, however, is that the transient signal for a single particle event is of the order of 100-150 μs.

The baseline is very different between the two datasets shown in FIGS. **11**A and **11**B. This difference can be attributed to the fact that a first cell detected should trip the higher threshold detector protection circuitry and activate ion defocusing. This is because the anticipated 2×105 Na ions per cell arrive in the period of approximately 100-150 μs, which corresponds to an average count rate exceeding 109 per second, is sufficient to trip the second threshold detector. In the absence of cells in the sample (data given in FIG. **11**B), the detector protection circuitry is not tripped. The ion optical defocusing appears to suppress ion transport by about a factor of 1000, accounting for the difference in the baseline data, but this is not intended to be a stable or reproducible (quantitative) defocusing factor.

Transient signals of 100-150 μs period, ascribed to MO7e introduction events, were observed at a frequency of about 5 to 6 per 10 milliseconds, or about 500 to 600 cells per second. This is consistent with the estimates made earlier, and with the estimate of 106 cells per 1 mL in the original sample (procedure step 4 of Feasibility Test 1, subsequently reduced to approximately 1 mL volume in step 9).

A notable inference taken from this experiment is that the high Na^+ signal anticipated for cells, or effects related to the change in mass distribution of the plasma ions as a result of the passage of a particle through the plasma, might provide a means to trigger the system upon a cell event. Further, it is feasible that the magnitude of the Na^+ signal (or signal of another element at high concentration in the cell), or the magnitude of effects related to the ion distribution change as a result of the particle's passage through the plasma, could be correlated with the physical size of the particle, which may be of importance in identifying target particles or distinguishing single particles from groups of particles.

The important conclusion of this experiment is that the transient signal is approximately 100-150 μs FWHM in duration. This has implications for design considerations to provide dynamic range. Further, it is evident that particles characterized by transient signals of this period can be introduced to the system at a rate of about 3000 per second (so that signal corresponding to a particle is present up to 50% of the time). Smaller cells, and smaller beads, should have shorter transients, and thus allow higher rate of introduction.

Feasibility Test 3: Comparison of Current FACS With the Current ICP-MS With Cell Injection, and Demonstration of Entire Cell Volatilization

The inventors have had an opportunity to compare directly the performance of a current FACS instrument to that of the ICP-MS instrument with cell injection described in Feasibility Test 1. Further, the test was configured to provide for tagging of intracellular proteins; if these internal tags can be detected, this implies that the entire cell and its contents were vaporized, atomized and ionized, rather than just vaporization of surface tags.

Because the ICP-MS instrument used for these experiments was not a simultaneous detector, the same (nanogold) tags could be used for each antigen, and immuno-tagging was performed in separate vials for each sample and antigen. Thus, each antigen for each sample was determined in a separate analysis. Samples were introduced to the ICP-MS as described in Feasibility Test 1.

Preparation of Samples for ICP-MS Analysis with Cell Injection

Materials

Human Monocyte Cell Lines:

MO7e parent line is a human megakaryocytic leukemia-derived cell. MO7e express CD33 antigen (67 kDa single chain transmembrane glycoprotein, myeloid cell surface antigen CD33 precursor (gp67)). Approximately 5000-10000 copies of CD33 antigen per cell.

MBA-I and MBA-4 are stable clones of MO7e transfected with p210 BCR/Abl expression plasmid.

HL-60 (ATCC cat # CCL-240), myeloid leukemia cell line used as antigen in production of anti-CD33 monoclonal antibodies.

Antibodies:

anti-CD33, mouse monoclonal, unconjugated. IgG 1 (mouse) isotype. Supplied at 2 mg/ml purified in PBS/BSA with 0.1% sodium azide (Immunotech Inc. Cat #1134)

anti-IgG2a, mouse, (BD PharMingen, cat #555571) (0.5 mg/ml stock)

anti-BCR antibody raised in rabbit (Cell Signaling Tech. Cat #3902), used at 1:25 for flow cytometry

Secondary antibodies: 2001 nanogold-anti-mouse IgG (NMI) and 2004 nanogold anti-rabbit Fab' (NRF) (Nanoprobes Inc.) used at (1:50) according to manufacturer's recommendation.

Buffers:

BD Biosciences FACS permeabilization solution 2 (cat #347692) PBS with Ca++/Mg++;

PBS/1% BSA

1% and 0.5% formalin prepared from 37% formalin; diluted in PBS 50 mM ammonium bicarbonate buffer, pH 8.0

Procedure:

Tubes were soaked in PBS/1% BSA for one hour.

Cells were pelleted at 1500 rpm(~200 g) 5 min, resuspended in 5 ml PBS and counted using a hemocytometer. Cell yield:

MO7e-1e6/ml

MBA-1-1e6/ml

MBA-4-1e6/ml

HL-60-1e6/ml

MO7e (tube #1) and HL-60 (tube #2) were stained live with anti-CD33 (1:50) on ice for 30 min; followed by one wash with PBS/BSA. Anti-mouse-IgG-Au (1:50) was added to the washed cell pellet for another 30 min on ice. Live stained cells were fixed in 1% formalin/PBS for 10 min RT and left in the fixative on ice over 48 hours.

MBA-I (tube #3), MBA-4 (tube #4) and MO7e (tube #5) were permeabilised and fixed in the FACS Permeabilization Solution 2 for 10 min at RT.

After one wash the cells were incubated in media with 10% FBS to block non-specific antigen sites for 15 min RT.

Permeabilized cells were treated with anti-BCR antibodies (1:25) (tubes #3,4,5) or with non-specific IgG (tubes #3°., 4°., 5°. w/o primary antibody) for 45 min RT. Secondary antibodies were added to washed cells anti-rabbit-IgG-Au (1:50) for 45 min RT.

Stained cells were washed twice prior to post-fixation in 0.5% formalin and kept in fridge over the weekend prior to MS analysis when the formalin was replaced with 50 mM ammonium bicarbonate.

Preparation of Samples for FACS Analysis (Carried Out Simultaneously With Above)

Materials

Antibodies:

anti-IgG 1-FITC mouse isotype, (BD PharMingen)

anti-CD45-FITC antibody raised in mouse (BD PharMingen) used at 1:50 for flow cytometry. CD45 is expressed on the surface of all human leukocytes. Used as a positive sample for FACS set-up.

Secondary fluorescent antibodies: anti-mouse IgG-FITC (BD PharMingen) and anti-rabbit-FITC (Biolab) used at (1:50)

Buffers:

BD Biosciences FACS permeabilization solution 2 (cat#347692)

PBS with Ca++/Mg++;

PBS/1% BSA

1% and 0.5% formalin prepared from 37% formalin; diluted in PBS 50 mM ammonium bicarbonate buffer, pH 8.0

Procedure

Cell preparation and primary antibody staining was done in parallel with samples for ICP-MS with 1e6 cells/ml/tube.

All procedures with fluorescent secondary antibody staining and cell washes were carried out in the dark on ice.

After the final PBS wash cells were resuspended in PBS (not formalin) and immediately processed by FACS (BD FACSCalibur).

Gates and settings were determined using the anti-CD45-FITC stained HL-60 as positive (R4) channel and isotype anti-mouse IgG-FITC stained HL-60 as negative (R3) channel.

Observations

The results for both the ICP-MS detection (shaded grey) and conventional FACS (white) are summarized in FIG. **12**.

Standard deviations for triplicate analyses by ICP-MS are shown by error bars; equivalent uncertainties for the FACS results were not provided.

Both the CD33 (surface markers on MO7e and HL60) and the BCR (internal marker in MO7e, MBA1 and MBA4) were determined by both FACS and ICP-MS detectors. This implies that the entire (permeabilized) cells and their contents were vaporized, atomized and ionized in the ICP-MS. Further, the FACS and ICP-MS results are largely in rather good agreement for both the surface and internal markers and for the procedural blanks.

We conclude from these results that FACS and ICP-MS detection (using the current un-optimized instrument) provide comparable results for single antigen assay. It is anticipated that the sensitivity of the ICP-MS detector will be improved as discussed above, and that incorporation of a simultaneous mass analyzer will permit high order multiplex assay. It is also evident that the MO7e, MBA1 and MBA4 cells used in these experiments were efficiently vaporized, atomized and ionized. This suggests that the optional in-line lysis device discussed above is not required for these or similar cells.

Feasibility Test 4: Production and Detection of Element-Tagged Beads

Another approach to multiplexed assay is to use different identifiable beads that immobilize antigens. The beads typically have capture affinity agents (e.g., antibodies) attached to their surface. After exposure to a sample, the bead-antigen complexes are typically exposed to a second affinity product (antibody, aptamer, etc.) which is tagged with an element or isotope as already discussed (herein, and in U.S. patent application Ser. No. 09/905,907, published under US 2002/0086441 on Jul. 4, 2002 and Ser. No. 10/614,115). The beads are distinguished by their elemental composition, which might be a surface element label, and encapsulated element label or an element label incorporated within the bead material. The identity of the bead can be associated with the

type of capture affinity agent attached to the bead or to the sample (e.g., beads with different element labels are exposed to different samples, or are placed in different wells of a 96- or 384- or 1536-well plate). Thus, detection of the secondary affinity product tag determines the presence of the antigen and the element composition (element label) of the bead indicates which antigen was captured or the sample in which it was captured. The method is modeled after U.S. Pat. No. 6,524,793, assigned to Luminex, and references therein.

The beads may be of any appropriate material (e.g., polystyrene, agarose, silica). Each bead may contain one or more affinity capture agents, and multiplexed assay of the antigens captured on the bead may be conducted. The element label incorporated in or on the bead may be a single element or isotope or, preferably, a combination of elements or isotopes. For example, if the dynamic range of the detector is three orders of magnitude and differences in signal levels of a factor of three are reliably detected, two element labels can be combined in different ratios to provide 63 distinguishable beads. Under the same conditions, 5 element labels can provide 32,767 distinguishable beads. With 5 orders of dynamic range and 5 element labels for which factors of three in signal can be reliably detected, 248,831 distinguishable beads can be constructed. It will be recognized that the beads can be manufactured to a size suitable for complete vaporization, atomization and ionization in the device used for that purpose (e.g., ICP). It will also be recognized that smaller beads are likely to provide shorter transient signals, and that accordingly the rate of particle introduction can be optimized for the particular beads used.

To demonstrate the viability of the method, stober silica particles having a diameter of about 150 nm were grown in various lanthanide (Ho, Tb, Tm) solutions. The lanthanide elements were incorporated into the silica particles. The silica particles (beads) were introduced serially to the ICP-MS instrument as described in Feasibility Test 1. Since the instrument used was not capable of simultaneous multielement analysis, the transient signals for the lanthanides and for silicon were measured separately for different beads.

FIGS. **13**A and **13**B show some of the data obtained. The data provided in FIG. **13**A shows the detection of Si^+, clearly indicating that the beads are vaporized, atomized and ionized. Data provided in FIG. **13**B show the detection of Tb^+ (for beads grown in Tb solution). Clearly, the Tb label is detected. If a mixture of beads having different lanthanide labels were sampled, the different lanthanide signals would identify the different beads. It is also evident that beads can be grown in solutions of mixed lanthanides (or other elements), and would incorporate the different elements, thus providing for a larger number of distinguishable beads as indicated above. The availability of a simultaneous analyzer would further allow simultaneous detection of the elements associated with the bead itself and also with the tag associated with a secondary affinity product that recognizes a captured antigen.

Therefore, elements within a bead can be detected (i.e., the bead is vaporized to its atomic components). Different combinations of element internal "labels" can be used to distinguish beads. If those beads carry different surface antibodies to bind different antigens, and those antigens are then recognized by another antibody containing a different element reporter tag, a multiplexed assay is enabled. Alternatively, the differently labeled beads can be used with the same surface antibodies, but with the different beads being applied to different samples (such as a 96 well plate), so that the signal associated with the labeled affinity product identifies the antigen concentration in the sample indicated by the signals corresponding to the bead composition. Numerous modifications, variations and adaptations may be made to the particular embodiments of the invention described above without departing from the scope of the invention, which is defined in the claims.

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What is claimed is:

1. A system for sequentially analyzing single cells in a sample by mass spectrometry,

wherein the sample comprises a plurality of tagged cells tagged with a plurality of tagged antibodies, wherein each of the plurality of tagged antibodies is specific for a different analyte, and wherein each of the plurality of tagged antibodies is tagged with an elemental tag comprising a lanthanide or noble metal;

wherein the system comprises:

a first device to vaporize, atomize, and ionize multiple elemental tags from a single first cell of the plurality of tagged cells and multiple elemental tags from a single second cell of the plurality of tagged cells; and

a second device to detect, by mass spectrometry, lanthanides and/or noble metals of the single first cell by

detecting a transient signal of the multiple vaporized, atomized, and ionized elemental tags of the single first cell, and lanthanides and/or noble metals of the single second cell by detecting a transient signal of the multiple vaporized, atomized, and ionized elemental tags of the single second cell, wherein the transient signal associated with the single first cell and the transient signal associated with the single second cell are detected sequentially.

2. The system of claim **1**, wherein the system is further configured to lyse the plurality of tagged cells prior to vaporizing, atomizing, and ionizing the multiple elemental tags from the single first cell.

3. The system of claim **2**, wherein the system is further configured to sequentially vaporize, atomize, and ionize fragments of the single first cell.

4. The system of claim **1**, wherein system is further configured to sequentially detect fragments of the single first cell.

5. The system of claim **1**, wherein at least one of the plurality of tagged antibodies is tagged using diethylenetriaminepentaacetic acid anhydride (DTPA), 1,4,7,10-tetraazacyclododecanetetraacetic acid (DOTA), or a derivative thereof.

6. The system of claim **1**, wherein each of the plurality of tagged antibodies is tagged with a distinct isotope.

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